

**USE OF PROTEIN BIOMOLECULAR TARGETS  
IN THE TREATMENT AND VISUALIZATION OF BRAIN TUMORS**

5 FIELD OF USE

10 The present invention relates to the use of proteins which are differentially expressed in  
primary brain tumor tissues, as compared to normal brain tissues, as biomolecular targets for  
brain tumor treatment therapies. Specifically, the present invention relates to the use of  
immunotherapeutic and immunoimaging agents which specifically bind to one or more of  
15 angiopoietin related protein 2 (ARP-2,) secreted protein acidic, rich in cysteine (SPARC,) c-met  
proto-oncogene (C-MET,) brevican (BEHAB,) CD-44 antigen (CD-44,) tetraspanin 3 (TSPN3,)  
pleiotrophin (PTN,) osteopontin (OPN,) vasoactive intestinal peptide receptor-2 (VIPR-2,) and  
receptor protein tyrosine phosphatase zeta (PTP $\zeta$ ) for the treatment and visualization of brain  
tumors in patients. The present invention also provides compounds and pharmaceutically  
acceptable compositions for administration.

BACKGROUND OF THE INVENTION

Brain Tumor Biology and Etiology

20 Brain tumors are considered to have one of the least favorable prognoses for long term  
survival: the average life expectancy of an individual diagnosed with a central nervous system  
(CNS) tumor is just eight to twelve months. Several unique characteristics of both the brain and  
its particular types of neoplastic cells create daunting challenges for the complete treatment and  
management of brain tumors. Among these are 1) the physical characteristics of the intracranial  
space, 2) the relative biological isolation of the brain from the rest of the body, 3) the relatively  
25 essential and irreplaceable nature of the organ mass, and 4) the unique nature of brain tumor  
cells.

30 First and foremost, the intracranial space and physical layout of the brain create  
significant obstacles to treatment and recovery. The brain is made of, primarily, astrocytes  
(which make up the majority of the brain mass, and serve as a scaffold and support for the  
neurons), neurons (which carry the actual electrical impulses of the nervous system), and a minor  
contingent of other cells such as insulating oligodendrocytes (which produce myelin). These cell  
types give rise to primary brain tumors (e.g., astrocytomas, neuroblastomas, glioblastomas,

Because of the relatively small volume of the skull cavity, minor changes in the volume of tissue in the brain can dramatically increase intracranial pressure, causing damage to the entire organ (i.e., “water on the brain”). Thus, even small tumors can have a profound and adverse affect on the brain’s function. In contrast, tumors in the relatively distensible abdomen may reach several pounds in size before the patient experiences adverse symptoms. The cramped physical location of the cranium also makes surgery and treatment of the brain a difficult and delicate procedure. However, because of the dangers of increased intracranial pressure from the tumor, surgery is often the first strategy of attack in treating brain tumors.

are useful in other types of tumors, from crossing into the brain. Thus, many therapies directed at the brain must be delivered directly into the brain cavity (e.g., by an Ommaya reservoir), or administered in elevated dosages to ensure the diffusion of an effective amount across the BBB.

25 destroy potential tumor-producing cells also produce unacceptable losses of healthy brain tissue. The retention of patient cognitive function while eliminating the tumor mass is another challenge to brain tumor treatment. Neoplastic brain cells are often pervasive, and travel throughout the entire brain mass. Thus, it is impossible to define a true “tumor margin,” unlike, for example, in lung or bladder cancers. Unlike reproductive (ovarian, uterine, testicular, prostate, etc.), breast, 30 kidney, or lung cancers, the entire organ, or even significant portions, cannot be removed to prevent the growth of new tumors. In addition, brain tumors are very heterogeneous, with

different cell doubling times, treatment resistances, and other biochemical idiosyncrasies between the various cell populations that make up the tumor. This pervasive and variable nature greatly adds to the difficulty of treating brain tumors while preserving the health and function of normal brain tissue.

5           Although current surgical methods offer considerably better post-operative life for patients, the current combination therapy methods (surgery, low-dosage radiation, and chemotherapy) have only improved the life expectancy of patients by one month, as compared to the methods of 30 years ago. Without effective agents to prevent the growth of brain tumor cells that are present outside the main tumor mass, the prognosis for these patients cannot be significantly improved. Although some immuno-affinity agents have been proposed and tested for the treatment of brain tumors, see, e.g., the tenascin-targeting agents described in U.S. Patent No. 5,624,659, these agents have not proven sufficient for the treatment of brain tumors. Thus, therapeutic agents which are directed towards new molecular targets, and are capable of specifically targeting and killing brain tumor cells, are urgently needed for the treatment of brain tumors.

**ARP-2 (Angiopoeitin related protein-2, Angiopoeitin like - 2 [ANGPTL-2])**

20           Angiopoeitin related protein-2 (ARP-2), is related to the angiopoietin family of proteins, that includes Ang-1 and Ang-2. Like members of the angiopoietin family, ARP-2 contains a coiled-coil domain in the amino terminal portion and a fibrinogen-like domain in the carboxyl terminal portion. However, ARP-2 has a low homology with Ang-1 and Ang-2 and unlike Ang-1 and Ang-2, ARP-2 does not bind to the Tie-2 receptor, nor does ARP-2 bind to the closely related Tie-1 receptor. Hence, ARP-2 is believed to be part of a newly identified family of proteins termed angiopoietin related proteins. Like the angiopoietins, ARP-2 is a member of the  
25           fibrinogen superfamily, which also includes the fibrinogens and lectins.

30           ARP-2 is a glycosylated, secretory protein that induces sprouting in endothelial cells, most likely through autocrine or paracrine signaling, and it is preferentially expressed in the blood vessels and muscle cells. Hence, ARP-2 mediates the differentiated state of endothelial cells or for vascular remodeling and development. ARP-2 has not heretofore been associated with brain tumors.

**SPARC (Secreted protein, acidic, cysteine-rich; osteonectin; basement membrane protein (bm) 40)**

Secreted protein acidic and rich in cysteine, SPARC or BM-40, is a member of the counter-adhesive family of proteins. It is a developmentally regulated, secreted glycoprotein expressed in fetal astrocytes, particularly during tissue remodeling, vessel morphogenesis, and in response to stress. It has been hypothesized that SPARC may affect cell migration and vascular morphogenesis either by directly interacting with extracellular matrix (ECM) proteins (such as collagens I, III, IV and V) or by initiating a receptor mediated signaling event that induces changes in cytoplasmic components associated with focal adhesions. SPARC has been found to bind directly to vitronectin, a multifunctional adhesive protein that is a component of the brain vascular basement membranes.

SPARC may indirectly affect cell migration and motility by regulating the expression of matrix metallo-proteases and by modulating the expression of other proteolytic enzymes (such as collagenase) that degrade the ECM. Increased SPARC expression has also been observed in two forms of low-grade malignant gliomas, in all grades of human astrocytic tumors, and in tumor cells invading adjacent brain at the tumor/brain interface. Hence, SPARC may be an astrocytoma invasion related gene that functions in connection with vitronectin to balance the modulation of cellular adhesion to the ECM and it may promote diffuse tumor cell infiltration into adjacent brain by affecting both tumor and endothelial cell-ECM interactions.

Because SPARC is also found in bone, dentine, and many normal and neoplastic human soft tissues it may also play a regulatory function in the control of such diverse processes as bone mineralization, cell shape, tissue remodeling or repair, cell migration, proliferation, and differentiation. SPARC is also synthesized, stored, and secreted by human blood platelets, binds to plasminogen, and enhances tissue plasminogen activator conversion of plasminogen to plasmin.

**c-MET (Met proto-oncogene tyrosine kinase, hepatocyte growth factor receptor [HGFR])**

c-MET is a member of the Hepatocyte Growth Factor Receptor (HGFR) family and a heterodimeric cellular receptor for Hepatocyte Growth Factor (HGF). c-MET contains a disulfide-linked  $\alpha$ -chain of 50-kDa (which is located in the extracellular domain,) a 145-kDa  $\beta$ -chain (which includes an extracellular region,) a transmembrane spanning domain, and an

intracellular tyrosine kinase domain that can be activated by autophosphorylation. Hence, HGFR is a subset of the protein tyrosine-kinase family of membrane-spanning, cell surface receptors.

The receptor-ligand pair, c-MET and HGF, function as a growth factor, regulating cell growth, migration, and morphogenesis, and hence, may play a role in neoplastic formation and metastasis. Upon HGF or macrophage stimulating protein (MSP) binding, the c-MET protein receptor goes through a conformational change wherein the intracellular tyrosine residues of the  $\beta$  subunit become phosphorylated at residue 1235, and a second messenger signal cascade is induced. This change activates c-MET's intracellular receptor kinase activity, which is important to the growth and differentiation of epithelial cells in normal and malignant tissues. c-MET has been identified in both normal brain and on glial tumors, and is thought to be determinant in the pathological processes of various malignancies. For instance, detailed studies have shown that glioblastoma multiforme (GBM), a highly malignant brain tumor of astrocytic origin, expresses c-MET, and this research suggests a role in tumor progression.

#### **BEHAB (Brain-enriched hyaluronan binding protein, brevican)**

BEHAB is a brain-specific, extracellular matrix protein, that is a member of the chondroitin sulfate proteoglycan (CSPG) family. BEHAB is expressed only in the CNS. Although its function is unclear, BEHAB is reported to bind to HA at the N-terminus, lectins at the C-terminus, and may mediate binding of other ECM components like tenascin. This suggests that BEHAB may play a role in cell-cell and cell-matrix interactions thereby maintaining the extracellular environment of the brain. It has been reported that the highest levels of expression of BEHAB is during brain development and at times and places where glial cells are highly motile, as in cases of brain injury or trauma. BEHAB expression is also unregulated in primary gliomas of the central nervous system, but not in tumors of non-glial origin. In surgical samples of human gliomas (including astrocytoma, oligodendroglioma, and glioblastoma tumors), BEHAB expression is consistently and dramatically increased over the level of expression in the normal brain. Hence, BEHAB expression correlates with an invasive phenotype that promotes gliogenesis by contributing to cell movement through the ECM.

#### **CD-44 antigen**

CD-44 is a single-path, type I transmembrane protein with extracellular domains that are flexibly linked to the transmembrane segment. CD-44 is a member of the cartilage link protein family and belongs to the hyaloadherin or link protein superfamily (LPSF). As other members of the LPS family, CD-44 can be extensively glycosylated and is typically decorated with glycosaminoglycans (e.g., chondroitin, heparin, and keratin sulfate). The genomic structure of CD-44 consists of 21 exons, at least 11 of which can be variably spliced (v1-v10), that are located in the membrane-proximal extracellular region. Alternative splicing of these exons give rise to a variety of CD-44 isoforms (at least 30 different isoforms have been characterized to date) that are widely distributed and expressed in a cell-specific manner. Among the most frequently occurring isoforms are CD-44H, expressed on hematopoietic cells, and CD-44E, expressed in epithelial cells. CD-44(H) has also been found to be expressed in lymphocytes, macrophages, erythrocytes, fibroblasts, epithelial and endothelial cells, and neurons. It is the predominant isoform in normal brain and neuroectoderm-derived tumors and is expressed on both normal astrocytes and oligodendrocytes as well on neoplastic astrocytes and glioblastomas.

The family of CD-44 proteins has been implicated in lymphocyte activation and homing, endothelial migration, and tumor cell metastasis. CD-44 is believed to be the major receptor for Hyaluronic acid (HA). CD-44/HA interactions underlie a wide spectrum of functions in embryonic morphogenesis and organogenesis, hematopoiesis, lymphocyte homing. CD-44 also mediates the attachment of glioma cells to chondroitin sulfate, types I and IV collagen, fibronectin laminin, vitronectin and Martrigel. This suggest that CD-44 may play a role in cell-cell and cell-matrix interactions, affecting the extracellular environment of the brain. Because HA is a major component of the brain ECM, and CD-44 is one of the principal cellular receptors of HA, CD-44 expression coincides with brain tumor growth and invasiveness.

**PTN (Pleiotrophin, heparin binding growth factor 8, neurite growth-promoting factor 1)**

Pleiotrophin or PTN, is a platelet-derived, growth factor inducible, member of the pleiotrophin family of proteins that includes midkine and retinoic acid-induced heparin-binding protein. It is a developmentally regulated, secreted cytokine that stimulates mitogenesis, angiogenesis, and neurite and glial process outgrowth guidance activities. During development PTN is expressed in the brain, intestine, muscle, skin, heart, lung and kidney. In the adult, PTN is found primarily in the brain in association with axonal tracts during active mitogenesis and

may therefore play an important role in the development and maintenance of the nervous system. It has been found to bind heparin, heparin sulfate proteoglycans, the extracellular matrix, and is also a natural ligand for receptor protein tyrosine phosphatase (RPTP), signaling through ligand dependant receptor inactivation of RPTP. Receptor mediated endocytosis occurs following PTN binding and may be disrupted by heparin.

PTN has also been found to have oncogenic properties, inducing malignant transformation and tumor growth and progression. It has been described as a proto-oncogene that is expressed in many human tumors and cell lines derived from human tumors. PTN is a mitogen for fibroblasts, epithelial and endothelial cells, stimulates plasminogen-activator production, can induce tube formation, and therefore can serve as a tumor angiogenesis factor.

#### **OPN (osteopontin, secreted phosphoprotein 1, bone sialoprotein-1)**

Osteopontin or OPN, is a member of the osteopontin family. It is a glycosylated sialoprotein that is heavily phosphorylated and expressed in a variety of cells including bone, kidney, placenta, nerve cells and macrophages, as well as T lymphocytes, epidermal and bone cells. OPN is a part of the mineralized bone matrix and may play a role in bone resorption, by facilitating the attachment of osteoclasts to the bone surface, and may be functionally important as an adhesive and chemotactic molecule for vascular cells. OPN is a secreted protein that binds tightly to hydroxyapatite, and hence, is important to cell matrix interactions. It has been observed to interact with the CD-44 homing receptor to physiologically induce macrophage chemotaxis, which may be a mechanism utilized by metastatic brain tumors in the process of dissemination.

OPN has been observed in the microvasculature of glioblastomas associated with VEGF expression and OPN mRNA has been found to be overexpressed in high grade and metastatic brain tumors. Hence, OPN expression correlates with the malignancy grade of gliomas.

#### **VIPR-2 (Vasoactive intestinal peptide receptor-2)**

Vasoactive intestinal polypeptide receptor II (VIPR-2), VPAC-2, is a member of the G-protein receptor family, which includes such members as the calcitonin, parathyroid hormone, secretin, glucagon and VIP-1 receptors. VIPR-2 is a seven-transmembrane spanning G protein-coupled receptor that responds to VIP by stimulating cAMP production. VIPR-2 is found in the

brain as well as peripheral tissues such as the pancreas, skeletal muscle, heart, lung, kidneys, stomach, adipocytes and the liver, and in various cells of the immune system. In the brain, VIPR-2 functions as a neuroendocrine hormone and neurotransmitter receptor, and is found in the thalamus, hippocampus, suprachiasmatic nucleus and hypothalamus.

5 VIPR-2 is encoded by a nucleotide sequence of approximately 2.8kb, which codes for a 438 amino acid sequence of approximately 48-64 kDa. The receptor-ligand pair, VIPR-2 and VIP, have various functions dependent upon the tissue where in they are located. VIP is a late-developing, 28 amino acid peptide that, along with its receptor, is widely distributed throughout the peripheral body, and plays a role in cardiovascular, reproductive, pulmonary, immune and gastrointestinal systems, to effect vasodilatation, bronchodilation, immunosuppression, hormonal secretion, and increased gastric motility. However, the cerebral cortex has one of the highest reported concentrations of VIP, localized to intrinsic neurons throughout all neocortical regions. In the brain, VIP and its receptor, have behavioral, electrophysiological, secretory, metabolic, vascular, and mitogenic effects. For instance, the receptor-ligand pair play a role in cortical differentiation, the relaying of sensory information to the cortex, and the regulation of morphogenic events by the release of diffusible signals from glial cells. VIPR-2 and VIP also play a role in the growth and differentiation of neuroblastomas.

20 **TSPAN3 (Tetraspanin 3, tetraspanin TM-4A)**

The Tetraspanin superfamily, is a family of approximately 20 integral membrane proteins that are broadly expressed in most human tissues including neural and bone marrow derived tissues. The family shares a common motif that includes four putative transmembrane domains (TM1-4), a small extracellular domain (EC1) of 20-27 amino acids, and a larger extracellular domain (EC2) between TMS3 and TMS4 of 70-130 amino acids. Two conserved features of tetraspanins are critical to their structure and function. First, charged residues are present in or near the TM domains, second, a cluster of cysteine residues is in the putative EC2 domain. Most of the tetraspanins are modified by N-glycosylation.

Many Tetraspanin proteins affect the regulation of cellular proliferation, motility, differentiation, development. In some cells, Tetraspanins may act as adapters in ultimeric complexes that link plasma membrane proteins, like integrins, into signaling complexes with



other signaling molecules (e.g., phosphatidylinositol 4-kinase) at the plasma membrane and play a role in integrin-mediated cell migration, metastasis and tumor cell invasion. A number of tetraspanins have also been discovered as tumor-associated proteins, including C-029, PETA-3/SFA-1, and SAS, which is amplified in a subset of sarcomas. Of the various TM4SF proteins, CD9, CD63, CD81, CD82, and CD151 are the most widely distributed. CD9 is expressed on 90% of non-T cell acute lymphoblastic leukemia cells and on 50% of chronic lymphocytic and acute myeloblastic leukemias. CD63 is also expressed in early stage melanomas.

### **Protein Tyrosine Phosphatase Receptor Zeta (PTP $\zeta$ )**

Vital cellular functions, such as cell proliferation and signal transduction, are regulated in part by the balance between the activities of protein kinases and protein phosphatases. These protein-modifying enzymes add or remove a phosphate group from serine, threonine, or tyrosine residues in specific proteins. Some tyrosine kinases (PTK's) and phosphatases (PTPase's) have been theorized to have a role in some types of oncogenesis, which is thought to result from an imbalance in their activities. There are two classes of PTPase molecules: low molecular weight proteins with a single conserved phosphatase domain such as T-cell protein-tyrosine phosphatase (PTPT; MIM 176887), and high molecular weight receptor-linked PTPases with two tandemly repeated and conserved phosphatase domains separated by 56 to 57 amino acids. Examples of this latter group of receptor proteins include: leukocyte-common antigen (PTPRC; MIM 151460) and leukocyte antigen related tyrosine phosphatase (PTPRF; MIM 179590).

Protein tyrosine phosphatase zeta (PTP $\zeta$ ) [also known as PTPRZ, HPTP-ZETA, HPTPZ, RPTP-BETA( $\beta$ ), or RPTPB] was isolated as a cDNA sequence by two groups in the early nineties. The complete cDNA sequence of the protein is provided in SEQ ID NO. 1, and the complete deduced amino acid sequence is provided in SEQ ID NO. 2. Splicing variants and features are indicated in the sequences. Levy et al. ("The cloning of a receptor-type protein tyrosine phosphatase expressed in the central nervous system" *J. Biol. Chem.* 268: 10573-10581, (1993)) isolated cDNA clones from a human infant brain step mRNA expression library, and deduced the complete amino acid sequence of a large receptor-type protein tyrosine phosphatase containing 2,307 amino acids.

Levy found that the protein, which they designated PTP- $\beta$  (PTP $\zeta$ ), is a transmembrane protein with 2 cytoplasmic PTPase domains and a 1,616-amino acid extracellular domain. As in

PTP- $\gamma$  (MIM 176886), the 266 N-terminal residues of the extracellular domain are have a high degree of similarity to carbonic anhydrases (see MIM 114880). The human gene encoding PTP $\zeta$  has been mapped to chromosome 7q31.3-q32 by chromosomal *in situ* hybridization (Ariyama et al., "Assignment of the human protein tyrosine phosphatase, receptor-type, zeta (PTPRZ) gene to chromosome band 7q31.3" *Cytogenet. Cell Genet.* 70: 52-54 (1995)). Northern blot analysis has shown that showed that PTP-zeta is expressed only in the human central nervous system. By in situ hybridization, Levy et al. (1993) localized the expression to different regions of the adult human brain, including the Purkinje cell layer of the cerebellum, the dentate gyrus, and the subependymal layer of the anterior horn of the lateral ventricle. Levy stated that this was the first mammalian tyrosine phosphatase whose expression is restricted to the nervous system. In addition, high levels of expression in the murine embryonic brain suggest an important role in CNS development.

Northern analysis has shown three splice variants: the extracellular proteoglycan phosphacan, which contains the full extracellular region of the protein, and the long ( $\alpha$ ) and short ( $\beta$ ) forms of the transmembrane phosphatase. The  $\beta$  form lacks the extracellular 860 aa long insert domain of the protein, therefore it is not glycosylated. PCR studies of the gene in rat genomic DNA indicated that there are no introns at the putative 5' and 3' splice sites or in the 2.6 kb segment which is deleted in the short transmembrane protein. The phosphatases and the extracellular proteoglycan have different 3'-untranslated regions. Additional alternative mRNA splicing is likely to result in the deletion of a 7 amino acid insert from the intracellular juxtamembrane region of both long and short phosphatase isoforms. Simultaneous quantitation of the three major isoforms indicated that the mRNA encoding phosphacan had the highest relative abundance in the CNS while that encoding the short phosphatase isoform was most abundant relative to the other PTP $\zeta$  variants in the PNS.

PTP $\zeta$  has only been found to be expressed in the nervous system. By in situ hybridization, it has been localized to different regions of the adult brain, including the Purkinje cell layer of the cerebellum, the dentate gyrus, and the subependymal layer of the anterior horn of the lateral ventricle. High levels of PTP $\zeta$  have been seen in regions of the brain where there is continued neurogenesis and neurite outgrowth, and it seems to play a role in morphogenesis and plasticity of the nervous system. Phosphacan immunoreactivity has been associated with

perineuronal nets around parvalbumin-expressing neurons in adult rat cerebral cortex. Neurons as well as astrocytes have been shown to express phosphacan.

The transmembrane forms of PTP $\zeta$  are expressed on the migrating neurons especially at the lamellipodia along the leading processes. PTP $\zeta$  is postulated to be involved in the neuronal migration as a neuronal receptor of pleiotrophin distributed along radial glial fibers. PTP $\zeta$  has been shown to be highly expressed in radial glia and other forms of glial cells that play an important role during development. The anti-PTP $\zeta$  staining localizes to the radial processes of these cells, which act as guides during neuronal migration and axonal elongation. The pattern of RPTP-zeta expression has also been shown to change with the progression of glial cell differentiation.

The three splicing variants of RPTP-zeta have been shown to have different spatial and temporal patterns of expression in the developing brain. The 9.5-kb and 6.4-kb transcripts, which encode the  $\alpha$  and  $\beta$  transmembrane protein tyrosine phosphatases, were predominantly expressed in glial progenitors located in the subventricular zone. The 8.4-kb transcript, which encodes the secreted chondroitin sulfate proteoglycan phosphacan, was expressed at high levels by more mature glia that have migrated out of the subventricular zone. The three transcripts have also been shown to be differentially expressed in glial cell cultures.

In knockout studies, PTP $\zeta$ -deficient mice were viable, fertile, and showed no gross anatomical alterations in the nervous system or other organs. Therefore, it was deduced that PTP $\zeta$  is not essential for neurite outgrowth and node formation in mice. The ultrastructure of nerves of the central nervous system in PTP $\zeta$ -deficient mice suggests a fragility of myelin. However, conduction velocity was not altered. The normal development of neurons and glia in was thought to indicate that PTP $\zeta$  function is not necessary for these processes in vivo, or that a loss of PTP $\zeta$  can be compensated for by other protein tyrosine phosphatases expressed in the nervous system.

Following CNS injury, robust induction of phosphatase forms of PTP $\zeta$  mRNA has been observed in areas of axonal sprouting, and of both phosphatases and phosphacan mRNAs in areas of glial scarring. This is thought to imply that the encoded proteins and the cell adhesion molecules and extracellular matrix proteins to which they bind may contribute to recovery from injury and perhaps also to the regulation of axonal regrowth in the nervous system. Following

peripheral nerve crush, all PTP $\zeta$  mRNAs, including phosphacan and the phosphatase variants with and without the 21 base insert, were observed to be significantly induced in the distal segments of the sciatic nerve with a time course that correlated well with the response of Schwann cells to this injury.

5       The extracellular domains of PTP $\zeta$  have been shown to be capable of binding to several cell adhesion molecules. Phosphacan, which is the shortest, secreted form of PTP $\zeta$ , containing the full extracellular region, previously was designated 3F8 and 6B4 chondroitin sulfate proteoglycan or 3H1 keratin sulfate proteoglycan depending on the glycosylation status. It is synthesized mainly by glia and binds to neurons and to the neural cell adhesion molecules Ng-CAM/L1, NCAM, TAG-1/axonin-1, to tenascin-C and R, to amphoterin and  
10       pleiotrophin/heparin-binding growth-associated molecule (HB-GAM) (amphoterin and pleiotrophin are heparin-binding proteins that are developmentally regulated in brain and functionally involved in neurite outgrowth). Binding of phosphacan to Ng-CAM/L1, NCAM, and tenascin-C (FNIII domain) is mediated by complex-type N-linked oligosaccharides on the proteoglycan. Phosphacan, shows saturable, reversible, high-affinity binding to fibroblast growth factor-2 (FGF-2). The interaction is mediated primarily through the core protein.  
15       Immunocytochemical studies have also shown an overlapping localization of FGF-2 and phosphacan in the developing central nervous system. The core protein of phosphacan may also regulate the access of FGF-2 to cell surface signaling receptors in nervous tissue.

20       The carbonic anhydrase (CAH) domain of PTP $\zeta$  has been shown to bind specifically to contactin. Contactin is a 140 kDa GPI membrane-anchored neuronal cell recognition protein expressed on the surface of neuronal cells. The CAH domain of RPTP zeta was shown to induce cell adhesion and neurite growth of primary tectal neurons, and differentiation of neuroblastoma cells. These responses were blocked by antibodies against contactin, demonstrating that contactin  
25       is a neuronal receptor for RPTP zeta. Caspr ((p190/Caspr, a contactin-associated transmembrane receptor) and contactin exist as a complex in rat brain and are bound to each other by means of lateral (cis) interactions in the plasma membrane. The extracellular domain of Caspr contains a neurophilin/coagulation factor homology domain, a region related to fibrinogen beta/gamma, epidermal growth factor-like repeats, neurexin motifs as well as unique PGY repeats found in a  
30       molluscan adhesive protein. The cytoplasmic domain of Caspr contains a proline-rich sequence capable of binding to a subclass of SH3 domains of signaling molecules. Caspr may function as a

signaling component of contactin, enabling recruitment and activation of intracellular signaling pathways in neurons. The role of the extracellular domains in neural adhesion and neurite growth induction was investigated by the use of fusion protein constructs. The results suggested that binding of glial PTP $\zeta$  to the contactin/Nr-CAM complex is important for neurite growth and neuronal differentiation.

PTP $\zeta$  was shown to bind to a heparin-binding growth factor midkine through the chondroitin sulfate portion of the receptor. The interactions of pleiotrophin (PTN) with the receptor in U373-MG cells was also studied. Pleiotrophin was shown to bind to the spacer domain. Results suggested that PTN signals through "ligand-dependent receptor inactivation" of PTP $\zeta$  and disrupts its normal roles in the regulation of steady-state tyrosine phosphorylation of downstream signaling molecules. PTN was shown to bind to and functionally inactivate the catalytic activity of PTP $\zeta$ . An active site-containing domain of PTP $\zeta$  both binds  $\beta$ -catenin and functionally reduces its levels of tyrosine phosphorylation when added to lysates of pervanadate-treated cells. In unstimulated cells, PTP $\zeta$  was shown to be intrinsically active, and thought to function as an important regulator in the reciprocal control of the steady-state tyrosine phosphorylation levels of  $\beta$ -catenin by tyrosine kinases and phosphatases.

Using the yeast substrate-trapping system, several substrate candidates for PTP $\zeta$  were isolated. The results indicated that GIT1/Cat-1 is a substrate molecule of PTP $\zeta$ . In addition, PTP $\zeta$  was shown to bind to the PSD-95/SAP90 family through the second phosphatase domain. Immunohistochemical analysis revealed that PTP $\zeta$  and PSD-95/SAP90 are similarly distributed in the dendrites of pyramidal neurons of the hippocampus and neocortex. Subcellular fractionation experiments indicated that PTP $\zeta$  is concentrated in the postsynaptic density fraction. These results suggested that PTP $\zeta$  is involved in the regulation of synaptic function as postsynaptic macromolecular complexes with PSD-95/SAP90.

Voltage-gated sodium channels in brain neurons were also found to associate with the membrane bound forms of PTP $\zeta$  and phosphacan. Both the extracellular domain and the intracellular catalytic domain of PTP $\zeta$  interacted with sodium channels. Sodium channels were tyrosine phosphorylated and were modulated by the associated catalytic domains of PTP $\zeta$ .

## SUMMARY OF THE INVENTION

The present invention provides novel methods and reagents for specifically targeting brain tumor neoplastic cells for both therapeutic and imaging purposes, by targeting brain tumor protein targets ( $T_{BT}$ ). These targets have been identified by the applicants as being overexpressed in brain tumors, and thus allow for the selective inhibition of cell function or selective marking for visualization with therapeutic or visualizing compositions which have a specific affinity for these protein targets. Each of angiopoietin related protein 2 (ARP-2,) secreted protein acidic, rich in cysteine (SPARC,) c-met proto-oncogene (C-MET,) brevican (BEHAB,) CD-44 antigen (CD-44,) tetraspanin 3 (TSPN3,) pleiotrophin (PTN,) osteopontin (OPN,) vasoactive intestinal peptide receptor-2 (VIPR-2,) and receptor protein tyrosine phosphatase zeta (PTP $\zeta$ ) [including the two novel isoforms PTP $\zeta$  SM1 and SM2], as the proteins are described below, have been identified as an independently useful protein target  $T_{BT}$ . In some preferred embodiments of the invention, either novel isoform PTP $\zeta$  SM1 or PTP $\zeta$  SM2 is the protein target  $T_{BT}$ . Thus, the aspects of the invention with relation to each of these  $T_{BT}$  are described generally as follows:

In a first aspect, the present invention provides  $T_{BT}$  affinity-based compounds and compositions useful in treating a brain tumor in a patient. The compositions and compounds of this aspect of the invention generally fall into two groups:  $T_{BT}$ -binding conjugate compounds, which comprise a cytotoxic moiety (C), which inhibits the growth of tumor cells; and  $T_{BT}$ -binding compound compositions in which the  $T_{BT}$  binding moiety alters the normal function of the  $T_{BT}$  in or around the tumor cell, thus inhibiting cell growth and/or function.

In a first group of embodiments of this aspect of the invention,  $T_{BT}$ -binding therapeutic conjugate compounds are provided. These compounds have the general formula  $\alpha(T_{BT})C$ , wherein  $\alpha(T_{BT})$  is one or more moieties which specifically binds to a  $T_{BT}$ , and C is one or more cytotoxic moieties. In preferred embodiments,  $\alpha(T_{BT})$  is an antibody or an antibody fragment. In particularly preferred embodiments,  $\alpha(T_{BT})$  is an antibody or an antibody fragment which elicits a reduced immune response when administered to a human patient. Preferred cytotoxic moieties for use in these embodiments of the invention include radioactive moieties, chemotoxic moieties, and toxin proteins. The invention also provides compositions comprising these  $T_{BT}$ -binding therapeutic conjugate compounds in a pharmaceutically acceptable carrier.

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invention be formulated into therapeutic compositions comprising the T<sub>BT</sub>-binding compound in a pharmaceutically acceptable carrier.

In a fourth aspect, the present invention provides methods of using the compounds and compositions of the third aspect of the invention to visualize a brain tumor in a patient. These methods generally comprise administering an effective amount of an imaging compound of the general formula  $\alpha(\text{T}_{\text{BT}})\text{I}$  in a pharmaceutically acceptable carrier to the patient, and then visualizing the imaging moieties of the compound. Administration of the imaging composition may be by any acceptable means. Intravascular administration of the imaging composition is preferred in these methods, although intrathecal administration is also preferred. Preferred methods of visualizing the imaging moieties of the compounds include radiographic imaging techniques (e.g., x-ray imaging and scintillation imaging techniques), positron-emission tomography, magnetic resonance imaging techniques, and direct or indirect (e.g., endoscopic) visual inspection.

Various particular embodiments of these aspects of the invention include:

A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{ARP}2)\text{C}$ , wherein  $\alpha(\text{ARP}2)$  is one or more moieties which specifically binds to a human angiopoietin related protein-2, and C is one or more cytotoxic moieties, and a pharmaceutically acceptable carrier.

A compound for the treatment of a brain tumor of the general formula  $\alpha(\text{ARP}2)\text{C}$ , wherein  $\alpha(\text{ARP}2)$  is one or more moieties which specifically binds to a human angiopoietin related protein-2, and C is one or more cytotoxic moieties.

A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{ARP}2)$ , wherein  $\alpha(\text{ARP}2)$  is one or more moieties which specifically binds to a human angiopoietin related protein-2, wherein the binding of  $\alpha(\text{ARP}2)$  alters the function of the angiopoietin related protein-2, and a pharmaceutically acceptable carrier.

A method for visualizing a brain tumor in a patient by first administering to a patient an effective amount of a composition comprising: a compound of the general formula  $\alpha(\text{ARP}2)\text{I}$ , wherein  $\alpha(\text{ARP}2)$  is one or more moieties which specifically binds to a human angiopoietin



related protein-2, and I is one or more imaging moieties and a pharmaceutically acceptable carrier, and then visualizing the imaging moieties of the compound.

A composition for the visualization of a brain tumor comprising a compound of the general formula  $\alpha(\text{ARP2})\text{I}$ , wherein  $\alpha(\text{ARP2})$  is one or more moieties which specifically binds to a human angiopoietin related protein-2, and I is one or more imaging moieties, and a pharmaceutically acceptable carrier.

A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{TSPAN3})\text{C}$ , wherein  $\alpha(\text{TSPAN3})$  is one or more moieties which specifically binds to a human tetraspanin 3, and C is one or more cytotoxic moieties, and a pharmaceutically acceptable carrier.

A compound for the treatment of a brain tumor of the general formula  $\alpha(\text{TSPAN3})\text{C}$ , wherein  $\alpha(\text{TSPAN3})$  is one or more moieties which specifically binds to a human tetraspanin 3, and C is one or more cytotoxic moieties.

A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{TSPAN3})$ , wherein  $\alpha(\text{TSPAN3})$  is one or more moieties which specifically binds to a human tetraspanin 3, wherein the binding of  $\alpha(\text{TSPAN3})$  alters the function of the tetraspanin 3, and a pharmaceutically acceptable carrier.

A composition for the treatment of a brain tumor comprising: a compound of the general formula  $\alpha(\text{TSPAN3})$ , wherein  $\alpha(\text{TSPAN3})$  is one or more moieties which specifically binds to a human tetraspanin 3, wherein the binding of  $\alpha(\text{TSPAN3})$  alters the function of the tetraspanin 3, and a pharmaceutically acceptable carrier.

A method for visualizing a brain tumor in a patient by first administering to a patient an effective amount of a composition comprising: a compound of the general formula  $\alpha(\text{TSPAN3})\text{I}$ , wherein  $\alpha(\text{TSPAN3})$  is one or more moieties which specifically binds to a human tetraspanin 3, and I is one or more imaging moieties, and a pharmaceutically acceptable carrier, and then visualizing the imaging moieties of the compound.

A composition for the visualization of a brain tumor comprising a compound of the general formula  $\alpha(\text{TSPAN3})\text{I}$ , wherein  $\alpha(\text{TSPAN3})$  is one or more moieties which specifically binds to a human tetraspanin 3, and I is one or more imaging moieties, and a pharmaceutically acceptable carrier.

A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{PTP}\zeta)\text{C}$ , wherein  $\alpha(\text{PTP}\zeta)$  is one or more moieties which specifically binds to a human PTP $\zeta$  selected from the group consisting of PTP $\zeta$  SM1 and PTP $\zeta$  SM2, further wherein  $\alpha(\text{PTP}\zeta)$  does not specifically bind to human PTP $\zeta$   $\alpha$ , human PTP $\zeta$   $\beta$ , or phosphacan, and C is one or more cytotoxic moieties, and a pharmaceutically acceptable carrier.

A compound for the treatment of a brain tumor of the general formula  $\alpha(\text{PTP}\zeta)\text{C}$ , wherein  $\alpha(\text{PTP}\zeta)$  is one or more moieties which specifically binds to a human PTP $\zeta$  selected from the group consisting of PTP $\zeta$  SM1 And PTP $\zeta$  SM2, further wherein  $\alpha(\text{PTP}\zeta)$  does not specifically bind to human PTP $\zeta$   $\alpha$ , human PTP $\zeta$   $\beta$ , or human phosphacan, and C is one or more cytotoxic moieties.

A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{PTP}\zeta)$ , wherein  $\alpha(\text{PTP}\zeta)$  is one or more moieties which specifically binds to a human PTP $\zeta$  selected from the group consisting of PTP $\zeta$  SM1 And PTP $\zeta$  SM2, further wherein  $\alpha(\text{PTP}\zeta)$  does not specifically bind to human PTP $\zeta$   $\alpha$ , human PTP $\zeta$   $\beta$ , or phosphacan, wherein the binding of  $\alpha(\text{PTP}\zeta)$  alters the function of the human PTP $\zeta$ , and a pharmaceutically acceptable carrier.

A composition for the treatment of a brain tumor comprising a compound of the general formula  $\alpha(\text{PTP}\zeta)$ , wherein  $\alpha(\text{PTP}\zeta)$  is one or more moieties which specifically binds to PTP $\zeta$  selected from the group consisting of PTP $\zeta$  SM1 And PTP $\zeta$  SM2, further wherein  $\alpha(\text{PTP}\zeta)$  does not specifically bind to human PTP $\zeta$   $\alpha$ , human PTP $\zeta$   $\beta$ , or phosphacan, wherein the binding of  $\alpha(\text{PTP}\zeta)$  alters the function of the PTP $\zeta$ , and a pharmaceutically acceptable carrier.

A method for visualizing a brain tumor in a patient by first administering to a patient an effective amount of a composition comprising: a compound of the general formula  $\alpha(\text{PTP}\zeta)\text{I}$ , wherein  $\alpha(\text{PTP}\zeta)$  is one or more moieties which specifically binds to a human PTP $\zeta$  selected from the group consisting of PTP $\zeta$  SM1 And PTP $\zeta$  SM2, further wherein  $\alpha(\text{PTP}\zeta)$  does not specifically bind to human PTP $\zeta$   $\alpha$ , human PTP $\zeta$   $\beta$ , or phosphacan, and I is one or more imaging moieties and a pharmaceutically acceptable carrier, and then visualizing the imaging moieties of the compound.

5 A composition for the visualization of a brain tumor comprising a compound of the general formula  $\alpha(\text{PTP}\zeta)\text{I}$ , wherein  $\alpha(\text{PTP}\zeta)$  is one or more moieties which specifically binds to a human  $\text{PTP}\zeta$  selected from the group consisting of  $\text{PTP}\zeta$  SM1 And  $\text{PTP}\zeta$  SM2, further wherein  $\alpha(\text{PTP}\zeta)$  does not specifically bind to human  $\text{PTP}\zeta$   $\alpha$ , human  $\text{PTP}\zeta$   $\beta$ , or phosphacan, and I is one or more imaging moieties, and a pharmaceutically acceptable carrier.

A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{SPARC})\text{C}$ , wherein  $\alpha(\text{SPARC})$  is one or more moieties which specifically binds to a human secreted protein, rich in cysteine, and C is one or more cytotoxic moieties, and a pharmaceutically acceptable carrier.

10 A compound for the treatment of a brain tumor of the general formula  $\alpha(\text{SPARC})\text{C}$ , wherein  $\alpha(\text{SPARC})$  is one or more moieties which specifically binds to a human secreted protein, rich in cysteine, and C is one or more cytotoxic moieties.

15 A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{SPARC})$ , wherein  $\alpha(\text{SPARC})$  is one or more moieties which specifically binds to a human secreted protein, rich in cysteine, wherein the binding of  $\alpha(\text{SPARC})$  alters the function of the secreted protein, rich in cysteine, and a pharmaceutically acceptable carrier.

20 A method for visualizing a brain tumor in a patient by first administering to a patient an effective amount of a composition comprising: a compound of the general formula  $\alpha(\text{SPARC})\text{I}$ , wherein  $\alpha(\text{SPARC})$  is one or more moieties which specifically binds to a human secreted protein, rich in cysteine, and I is one or more imaging moieties, and a pharmaceutically acceptable carrier, and then visualizing the imaging moieties of the compound.

25 A composition for the visualization of a brain tumor comprising a compound of the general formula  $\alpha(\text{SPARC})\text{I}$ , wherein  $\alpha(\text{SPARC})$  is one or more moieties which specifically binds to a human secreted protein, rich in cysteine, and I is one or more imaging moieties, and a pharmaceutically acceptable carrier.

30 A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{c-MET})\text{C}$ , wherein  $\alpha(\text{c-MET})$  is one or more moieties which specifically binds to a human c-MET oncogene product, and C is one or more cytotoxic moieties and a pharmaceutically acceptable carrier.

5 A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{c-MET})$ , wherein  $\alpha(\text{c-MET})$  is one or more moieties which specifically binds to a human c-MET oncogene product, wherein the binding of  $\alpha(\text{TSPAN3})$  alters the function of the c-MET oncogene product, and a pharmaceutically acceptable carrier.

10 A method for visualizing a brain tumor in a patient by first administering to a patient an effective amount of a composition comprising: a compound of the general formula  $\alpha(\text{c-MET})\text{I}$ , wherein  $\alpha(\text{c-MET})$  is one or more moieties which specifically binds to a human c-MET oncogene product, and I is one or more imaging moieties and a pharmaceutically acceptable carrier, and then visualizing the imaging moieties of the compound.

15 A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{CD44})\text{C}$ , wherein  $\alpha(\text{CD44})$  is one or more moieties which specifically binds to a human CD44 antigen, and C is one or more cytotoxic moieties, and a pharmaceutically acceptable carrier.

20 A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{CD44})$ , wherein  $\alpha(\text{CD44})$  is one or more moieties which specifically binds to a human CD44 antigen, wherein the binding of  $\alpha(\text{CD44})$  alters the function of the CD44 antigen, and a pharmaceutically acceptable carrier.

25 A method for visualizing a brain tumor in a patient by first administering to a patient an effective amount of a composition comprising: a compound of the general formula  $\alpha(\text{CD44})\text{I}$ , wherein  $\alpha(\text{CD44})$  is one or more moieties which specifically binds to a human CD44 antigen, and I is one or more imaging moieties, and a pharmaceutically acceptable carrier, and then visualizing the imaging moieties of the compound.

30 A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{VIPR2})\text{C}$ , wherein  $\alpha(\text{VIPR2})$  is one or more moieties which specifically binds to a human vasoactive intestinal peptide receptor-2, and C is one or more cytotoxic moieties and a pharmaceutically acceptable carrier.

35 A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{VIPR2})$ , wherein  $\alpha(\text{VIPR2})$  is one or more moieties which specifically binds to a human vasoactive intestinal peptide receptor-2, wherein

the binding of  $\alpha$ (VIPR2) alters the function of the vasoactive intestinal peptide receptor-2, and a pharmaceutically acceptable carrier.

A method for visualizing a brain tumor in a patient by first administering to a patient an effective amount of a composition comprising: a compound of the general formula  $\alpha$ (VIPR2)I, wherein  $\alpha$ (VIPR2) is one or more moieties which specifically binds to a human tetraspanin 3, and I is one or more imaging moieties, and a pharmaceutically acceptable carrier, and then visualizing the imaging moieties of the compound.

A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha$ (OPN)C, wherein  $\alpha$ (OPN) is one or more moieties which specifically binds to a human osteopontin, and C is one or more cytotoxic moieties and a pharmaceutically acceptable carrier.

A compound for the treatment of a brain tumor of the general formula  $\alpha$ (OPN)C, wherein  $\alpha$ (OPN) is one or more moieties which specifically binds to a human tetraspanin 3, and C is one or more cytotoxic moieties.

A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha$ (OPN), wherein  $\alpha$ (OPN) is one or more moieties which specifically binds to a human osteopontin, wherein the binding of  $\alpha$ (OPN) alters the function of the osteopontin, and a pharmaceutically acceptable carrier.

A method for visualizing a brain tumor in a patient by first administering to a patient an effective amount of a composition comprising: a compound of the general formula  $\alpha$ (OPN)I, wherein  $\alpha$ (OPN) is one or more moieties which specifically binds to a human osteopontin, and I is one or more imaging moieties, and a pharmaceutically acceptable carrier, and then visualizing the imaging moieties of the compound.

A composition for the visualization of a brain tumor comprising a compound of the general formula  $\alpha$ (OPN)I, wherein  $\alpha$ (OPN) is one or more moieties which specifically binds to a human osteopontin, and I is one or more imaging moieties, and a pharmaceutically acceptable carrier.

A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha$ (PTN)C, wherein  $\alpha$ (PTN) is one or more

moieties which specifically binds to a human pleiotrophin, and C is one or more cytotoxic moieties, and a pharmaceutically acceptable carrier.

A compound for the treatment of a brain tumor of the general formula  $\alpha(\text{PTN})\text{C}$ , wherein  $\alpha(\text{PTN})$  is one or more moieties which specifically binds to a human pleiotrophin, and C is one or more cytotoxic moieties.

A method to treat a brain tumor by administering a therapeutic amount of a composition comprising a compound of the general formula  $\alpha(\text{PTN})$ , wherein  $\alpha(\text{PTN})$  is one or more moieties which specifically binds to a human pleiotrophin, wherein the binding of  $\alpha(\text{PTN})$  alters the function of the pleiotrophin, and a pharmaceutically acceptable carrier.

A method for visualizing a brain tumor in a patient by first administering to a patient an effective amount of a composition comprising: a compound of the general formula  $\alpha(\text{PTN})\text{I}$ , wherein  $\alpha(\text{PTN})$  is one or more moieties which specifically binds to a human pleiotrophin, and I is one or more imaging moieties, and a pharmaceutically acceptable carrier, and then visualizing the imaging moieties of the compound.

A composition for the visualization of a brain tumor comprising a compound of the general formula  $\alpha(\text{PTN})\text{I}$ , wherein  $\alpha(\text{PTN})$  is one or more moieties which specifically binds to a human pleiotrophin, and I is one or more imaging moieties, and a pharmaceutically acceptable carrier.

Brain tumors are known to be relatively heterogeneous, and thus all patients may not respond the same to a particular protein target treatment. Thus, in addition to the independent uses of each of the  $\text{T}_{\text{BT}}$  protein targets as described above, the invention also provides, in yet another aspect, combination therapeutic and/or visualization agents, compositions, and methods. These combination embodiments of the invention may utilize as brain tumor protein targets any two or more of the identified targets angiopoietin related protein 2 (ARP-2,) secreted protein acidic, rich in cysteine (SPARC,) c-met proto-oncogene (c-MET,) brevican (BEHAB,) cd-44 antigen (CD-44,) tetraspanin 3 (TSPN3,) pleiotrophin (PTN,) osteopontin (OPN,) vasoactive intestinal peptide receptor-2 (VIPR-2,) and receptor protein tyrosine phosphatase zeta (PTP $\zeta$ ) [including the two novel isoforms PTP $\zeta$  SM1 and SM2]. In some preferred embodiments, the brain tumor protein targets are selected from the group consisting of angiopoietin related protein 2 (ARP-2,) c-met proto-oncogene (c-MET,) cd-44 antigen (CD-44,) tetraspanin 3 (TSPN3,)

osteopontin (OPN,) and receptor protein tyrosine phosphatase zeta (PTP $\zeta$ ). Embodiments of the combination aspect of the invention may target a group of proteins from the identified targets with similar compartmentalization characteristics. Thus, the combination aspects may target two or more secreted proteins from the group angiopoietin related protein 2 (ARP-2,) secreted protein acidic, rich in cysteine (SPARC,) brevican (BEHAB,) pleiotrophin (PTN,) secreted forms of receptor protein tyrosine phosphatase zeta (PTP $\zeta$ ) [including the novel isoform PTP $\zeta$  SM1]. Or, the combination aspects may target one or more of the extracellular matrix binding proteins secreted protein acidic, rich in cysteine (SPARC,) and/or brevican (BEHAB,) with one or more of the other identified protein targets. Or, the combination aspects may target one or more of the membrane-bound proteins from the group c-met proto-oncogene (c-MET,) cd-44 antigen (CD-44,) tetraspanin 3 (TSPN3,) osteopontin (OPN,) vasoactive intestinal peptide receptor-2 (VIPR-2,) and membrane bound forms of receptor protein tyrosine phosphatase zeta (PTP $\zeta$ ) [including the novel isoform PTP $\zeta$  SM2].

In preferred embodiments, at least one of the protein targets is selected from the proteins angiopoietin related protein 2 (ARP-2,) tetraspanin 3 (TSPN3,) and receptor protein tyrosine phosphatase zeta (PTP $\zeta$ ) [including the two novel isoforms PTP $\zeta$  SM1 and SM2]. In other preferred embodiments, one of these proteins and another protein selected from the secreted group, the extracellular matrix group, or the membrane-bound group. In another group of preferred embodiments are combination aspects targeting two or more of angiopoietin related protein 2 (ARP-2,) tetraspanin 3 (TSPN3,) and receptor protein tyrosine phosphatase zeta (PTP $\zeta$ ) [including the two novel isoforms PTP $\zeta$  SM1 and SM2]. Another group of preferred embodiments are combination aspects which target angiopoietin related protein 2 (ARP-2,) and one or more proteins selected from the group secreted protein acidic, rich in cysteine (SPARC,) c-met proto-oncogene (C-MET,) brevican (BEHAB,) CD-44 antigen (CD-44,) tetraspanin 3 (TSPN3,) pleiotrophin (PTN,) osteopontin (OPN,) vasoactive intestinal peptide receptor-2 (VIPR-2,) and receptor protein tyrosine phosphatase zeta (PTP $\zeta$ ) [including the two novel isoforms PTP $\zeta$  SM1 and SM2]. Another group of preferred embodiments are combination aspects which target tetraspanin 3 (TSPN3,) and one or more proteins selected from the group secreted protein acidic, rich in cysteine (SPARC,) c-met proto-oncogene (C-MET,) brevican (BEHAB,) CD-44 antigen (CD-44,) angiopoietin related protein 2 (ARP-2,) pleiotrophin (PTN,)

osteopontin (OPN,) vasoactive intestinal peptide receptor-2 (VIPR-2,) and receptor protein tyrosine phosphatase zeta (PTP $\zeta$ ) [including the two novel isoforms PTP $\zeta$  SM1 and SM2]. Another group of preferred embodiments are combination aspects which target receptor protein tyrosine phosphatase zeta (PTP $\zeta$ ) [including the two novel isoforms PTP $\zeta$  SM1 and SM2] and one or more proteins selected from the group angiopoietin related protein 2 (ARP-2,) secreted protein acidic, rich in cysteine (SPARC,) c-met proto-oncogene (C-MET,) brevican (BEHAB,) CD-44 antigen (CD-44,) tetraspanin 3 (TSPN3,) pleiotrophin (PTN,) osteopontin (OPN,) and vasoactive intestinal peptide receptor-2 (VIPR-2.) As pleiotrophin (PTN) is a known ligand of PTP $\zeta$ , another preferred embodiment of the combination aspects of the invention utilizes these proteins as targets, either alone or in combination with one or more of the other identified targets.

In yet another aspect, the present invention provides two novel splicing isoforms of PTP $\zeta$ , shown to be expressed in brain tissue. These novel isoforms, PTP $\zeta$  SM1 and PTP $\zeta$  SM2, described in more detail below, differ in structure from the three known isoforms heretofore disclosed. PTP $\zeta$  SM1 comprises the amino acids encoded by the first nine exons of PTP $\zeta$  -  $\alpha$ , with three unique additional carboxy terminal amino acids encoded by additional 3' mRNA sequence from the intron of the gene between exons nine and ten. The mRNA for PTP $\zeta$  SM2 comprises all exons of PTP $\zeta$  -  $\alpha$ , with a 116 nucleotide insertion, in the correct reading frame, in the mRNA sequence between exons 23 and 24, from the intron of the gene between exons 23 and 24. Thus, embodiments of this aspect of the invention include the mature proteins of PTP $\zeta$  splice variants SM1 or SM2, and nucleic acids encoding these novel splice variants, as well proteins with significant homology to the splice variants.

Thus, in one group of embodiments of this aspect, the invention provides nucleic acid polymers comprising the sequence of nucleotides 148 to 1272 of SEQ ID NO. 1, the complement of nucleotides 148 to 1272 of SEQ ID NO. 1, nucleotides 148 to 7209 of SEQ ID NO. 3, or the complement of nucleotides 148 to 7209 of SEQ ID NO. 3. In another group of embodiments of this aspect, the invention provides polypeptides comprising the amino acid sequence of SEQ ID NO. 2 or the amino acid sequence of SEQ ID NO. 4.

In an additional related aspect, the invention provides polypeptides comprising a distinctive portion of the amino acid sequence of SEQ ID NO. 2 or SEQ ID NO. 4. Such peptides are useful for the production of antibodies against the PTP $\zeta$  SM1 or SM2 splicing variants.



Preferably, these polypeptides comprise a portion of the amino acid sequence of SEQ ID NO. 2 or SEQ ID NO. 4 which is at least 6, more preferably at least 8, more preferably at least 10, more preferably at least 15, and most preferably at least 20 amino acids in length. In some preferred embodiments of this aspect of the invention, the polypeptides comprise the three unique terminal amino acids of PTP $\zeta$  SM1 after exon 9. In other preferred embodiments, the polypeptides comprise a portion of the unique exon 23a of PTP $\zeta$  SM2, wherein the portion is preferably at least 3 amino acids in length, more preferably at least 6 amino acids in length, more preferably at least 9 amino acids in length, and most preferably at least 15 amino acids in length.

In an additional related aspect, the invention also provides affinity reagents which specifically bind to PTP $\zeta$  splice variants SM1 or SM2, but do not bind to the other known splice variants of PTP $\zeta$  (e.g.,  $\alpha$ ,  $\beta$ , or phosphacan forms). In preferred embodiments these affinity reagents are antibodies or antibody fragments.

In an additional related aspect, the invention also provides nucleic acid sequences encoding the PTP $\zeta$  splice variants SM1 or SM2. The invention also encompasses nucleic acid probes which hybridize to the mRNA encoding PTP $\zeta$  splice variants SM1 or SM2, but not mRNA encoding other known splice variants of PTP $\zeta$ .

#### BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1: A diagram of the three known splicing variant isoforms of PTP $\zeta$ . The approximate position of the domains of the isoforms is indicated underneath the isoforms, as well as the approximate exon size (for size reference, exon 12 is 3.6 kilobases.) Isoform PTP $\zeta$  -  $\alpha$  is the full length isoform, which contains the primary amino acid sequence aa 25-2314 of SEQ ID NO. 2 (aa 1-24 are a signal polypeptide). In Isoform PTP $\zeta$  -  $\beta$ , aa 755-1614 are missing. Isoform PTP $\zeta$  -S (phosphacan), is a secreted isoform which comprise the extracellular domains of PTP $\zeta$  -  $\alpha$ , in which the transmembrane and cytosol domains are missing.

FIGURE 2: A diagram of the two newly discovered splicing variant isoforms of PTP $\zeta$ . The approximate position of the domains of the isoforms is indicated underneath the

isoforms, as well as the approximate exon size (for size reference, exon 12 is 3.6 kilobases.) SM 1 fails to splice correctly after the 9<sup>th</sup> exon, yielding an mRNA with tow extra codons followed by a stop codon after the normal terminus of exon 9. SM 2 contains a 116 nucleotide insertion from between exons 23 &24.

- 5 FIGURE 3: A diagram comparing the three known PTP $\zeta$  isoforms with the two novel isoforms.

#### DETAILED DESCRIPTION OF THE INVENTION

Applicants have identified several brain tumor protein targets and genes which are differentially regulated between brain cancer tissue (glioblastoma) and normal brain tissue. Applicants have performed differential cloning between cancerous and normal brains and have identified the brain tumor protein target genes by DNA sequence analysis. Based on the observation in other diseases, particularly other cancers, in which overexpressed genes can contribute to the pathology of the disease, these overexpressed genes and their protein products mediate the initiation and progression of brain tumors. Thus, the overexpressed brain tumor protein targets, which are presented on the cell surface, provide excellent targets for immunotherapeutic agents which either deliver cytotoxic agents to directly promote tumor cell death, or which alter the function of the brain tumor protein targets to inhibit the normal physiology of the tumor cell. In addition, immunoimaging agents targeted to the brain tumor protein targets may be utilized to visualize the tumor mass either in diagnostic methods (e.g., magnetic resonance imaging (MRI) or radiography), or in surgery (e.g., by the use of optically visual dye moieties in the immunoimaging agent).

Applicants have identified the brain tumor protein targets by a direct examination of the expression level of genes in actual tumor cells. These samples provide a more accurate and realistic picture of tumor cell biology, especially on the detailed transcriptome level, than animal models or established cell tissue culture cell lines. Several groups have found that cell lines established from astrocytomas and other cell lines do not exhibit expression patterns which reflect the actual expression of the original tumor. For instance, Schreiber, et. al., "Primary brain tumors differ in their expression of octamer deoxyribonucleic acid-binding transcription factors from long-term cultured glioma cell lines." *Neurosurgery* 34: 129-35 (1994), showed that nervous system-specific transcription factors known as N-Oct proteins are differentially

expressed in human neuroblastoma and glioblastoma cell lines in vitro. However, when these results were compared to freshly isolated human primary and metastatic brain tumors, of the five astrocytomas and three glioblastomas analyzed, all but two tumors displayed the complete N-Oct protein profile, irrespective of histopathological tumor grade. Similarly, Eberle, et al., "The expression of angiogenin in tissue samples of different brain tumors and cultured glioma cells." *Anticancer Res* 20: 1679-84 (2000), could show that angiogenin is detectable in different kinds of intracranial tumor tissue samples. Although angiogenin could be detected in primary cultivated glioma cells, it was not detected in the permanent cell lines. Finally, Hartmann, et al., "The rate of homozygous CDKN2A/p16 deletions in glioma cell lines and in primary tumors." *Int J Oncol* 15: 975-82 (1999), showed that the rate of homozygous deletions of CDKN2A/p16 is variable between different tumor entities, but the rate of deletions is higher in established cell lines in comparison with primary tumors. Hartmann hypothesized that such incongruity may reflect statistical sampling errors, true differences depending on tissue derivatization and CDKN2A/p16 loss under selective pressure in tissue culture. After comparing established cell lines derived from human glioblastomas and their corresponding primary tumors by multiplex PCR methodology, they found that in 2 of 11 cases (18%) the primary tumor had no p16 alteration whereas the corresponding cell lines had a homozygous p16 deletion, and that CDKN2A/p16 was lost already in the earliest passages of the cell lines. Thus, Hartmann concluded that the deletion was the result of selective cell-culture pressures in many cases.

These inconsistent results arise because the tumor tissue samples are obtained from their native milieu, without allowing them the opportunity to alter their gene expression levels in response to artificial environmental stimuli. As recently reported by the Brain Tumor Progress Review group of the National Cancer Institute in November, 2000, conventionally used glioblastoma cell lines contain genetic and gene expression alterations that are well defined and do not necessarily reflect the primary tumors from which they were derived. In addition, these cell lines are highly homogenous, unlike a primary brain tumor. Therefore, data derived solely from a cell line cannot reliably reflect the biology, heterogeneity, or therapeutic response of a primary brain tumor.

Applicants obtained tumor tissue, snap frozen in the operation hall from unknown patients, which was confirmed as glioblastoma grade IV by neuropathology. These tissues served as the experimental sample. Human whole brain tissue (Clontech Laboratories, Palo

Alto, USA) served as control sample. Poly-A<sup>+</sup> RNA prepared from the cells was converted into double-stranded cDNA (dscDNA).

Briefly, the ds-cDNA's from control and disease states were subjected to kinetic re-annealing hybridization during which normalization of transcript abundances and enrichment for differentially expressed transcripts (i.e., subtraction) occurs. Normalized-subtracted ds-cDNAs were cloned into a plasmid vector, a large number of recombinant bacterial clones were picked, and their recombinant inserts were isolated by PCR. High-density cDNA arrays of those PCR products were screened with cDNA probes derived from the original control and disease states. Thus, only clones displaying a significant transcriptional induction and/or repression were sequenced and carried forward for massive expression profiling using a variety of temporal, spatial and disease-related probe sets.

The selected PCR products (fragments of 200-2000 bp in size) from clones showing a significant transcriptional induction and/or repression were sequenced and functionally annotated in AGY's proprietary database structure (See WO01/13105). Because large sequence fragments were utilized in the sequencing step, the data generated has a much higher fidelity and specificity than other approaches, such as SAGE. The resulting sequence information was compared to public databases using the BLAST (blastn) and tblastx algorithm. The results are listed in Table 1, below:

**TABLE 1**

PROTEIN	RELATIVE EXPRESSION LEVEL	NUMBER OF CLONES ISOLATED (out of 20,000)
ARP2	~2 times	13

SPARC	~2 – 5.6 times	100
CMET	~1.2 – 2.5 times	30
CD 44	~2.3 – 3.0 times	6
BEHAB	~2 – 6 times	180
TSPAN3	~2.0 – 3.0 times	7
VIPR2	~3.0 times	3
OPN	~2.0 – 3.0 times	19
PTN	~1.8 – 2.6 times	26
PTP $\zeta$	~2.0 – 4.0 times	20

As one of skill in the art will appreciate from this data, each of these proteins is individually useful as a target for the treatment and/or imaging of brain tumors.

#### Characteristics of Protein Targets Utilized in the Invention

##### ARP2

Given the experiments described above, and the results of Table 1, ARP-2 was selected as a target for selective immuno-therapeutic agents in targeting and/or imaging brain tumors.

The mature protein consists of 493 amino acids and contains two potential consensus glycosylation sites. The complete cDNA sequence encoding ARP-2 is provided in SEQ ID NO. 7, and the complete amino acid sequence of ARP-2 is provided in SEQ ID NO. 8. ARP-2 is a 64 kDa, single chain, acidic, angiopoietin-like protein that includes multiple functional domains, such as a hydrophobic signal sequence from amino acids 1-21 (which is typical of secreted proteins), a coiled-coil domain at the amino terminal end from approximately amino acid sequences 22-274, and a fibrinogen-like domain, from approximately about residues 275 through 493. Two major isoforms have been observed, one 2.4 Kb in size and the other about 4 Kb. Both forms are abundant in heart, small intestine, spleen and stomach.

As used herein, a compound that specifically binds to ARP-2 is any compound (such as an antibody) that has a binding affinity for any naturally occurring isoform, splice variant, or polymorphism of ARP-2, explicitly including the isoforms described herein. As one of ordinary skill in the art will appreciate, such “specific” binding compounds (e.g., antibodies) may also

bind to other closely related proteins that exhibit significant homology (such as greater than 90% identity, more preferably greater than 95% identity, and most preferably greater than 99% identity) with the amino acid sequence of ARP-2. Such proteins include truncated forms or domains of ARP-2, and recombinantly engineered alterations of ARP-2. For example, a portion of SEQ ID NO. 8 may be engineered to include a non-naturally occurring cysteine for cross linking to an immunoconjugate protein, as described.

In general, it is preferred that the antibodies utilized in the compositions and methods of the invention bind to the fibrinogen domain but need not be restricted to this domain. The antibody may bind to the extracellular region of ARP-2. It is to be noted that antibodies which bind to this secreted protein are useful in the invention as cytotoxic delivery agents, as well as functional inhibition agents, as one of ordinary skill would expect that the concentration of ARP-2 would be increased adjacent the tumor cells which, due to the need for vascularization, over-express the protein.

When raising antibodies to ARP-2, the entire protein (either the unsecreted precursor or the secreted protein), or a portion thereof, may be utilized. For instance, the carboxyl-terminal fibrinogen like domain, or any portion of the amino-terminal coiled-coil domain may be utilized. For instance, amino acids 22-274, which make up the fibrinogen like domain, may be used. Larger ARP-2 proteins and domains may be produced utilizing any suitable recombinant vector/protein production system, such as the baculovirus transfection system outlined below, after being amplified from a fetal brain cDNA library (as available from, e.g., Clontech, Palo Alto, CA) or another suitable source.

When utilizing an entire protein, or a larger section of the protein, antibodies may be raised by immunizing the production animal with the protein and a suitable adjuvant (e.g., Freund's, Freund's complete, oil-in-water emulsions, etc.). In these cases, the ARP-2 protein (or a portion thereof) can serve as the ARP-2 antigen. When a smaller peptide is utilized, it is advantageous to conjugate the peptide with a larger molecule to make an immunostimulatory conjugate for use as the ARP-2 antigen. Commonly utilized conjugate proteins that are commercially available for such use include bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). In order to raise antibodies to particular epitopes, peptides derived from the full ARP-2 sequence may be utilized. Preferably, one or more 8-30 aa peptide portions of the EC domain of ARP-2 are utilized, with peptides in the range of 10-20 being a more economical

choice. Custom-synthesized peptides in this range are available from a multitude of vendors, and can be order conjugated to KLH or BSA. Alternatively, peptides in excess of 30 amino acids may be synthesized by solid-phase methods, or may be recombinantly produced in a suitable recombinant protein production system. In order to ensure proper protein glycosylation and processing, an animal cell system (e.g., Sf9 or other insect cells, CHO or other mammalian cells) is preferred. Other information useful in designing an antigen for the production of antibodies to ARP-2 may be deduced by those of skill in the art by homology analysis of SEQ ID NO. 8.

The fibrinogen domain of human ARP-2 is hypothesized to interact with one or more an unknown receptor for the purposes of angiogenesis. The interaction of ARP-2 to these molecules may be through either of the aforementioned structural motifs. Thus, in alternative embodiments of the compositions and methods of the invention, antibody moieties are utilized which bind to ARP-2 at a site on the protein that alters the binding of an extracellular molecule to ARP-2. Such ARP-2 activity altering antibodies may be utilized in therapeutic compositions in an unconjugated form (e.g., the antibody in an acceptable pharmaceutical carrier), or may be conjugated to either a therapeutic moiety (creating a double-acting therapeutic agent) or an imaging moiety (creating a dual therapeutic/imaging agent).

Selection of antibodies which alter (enhance or inhibit) the binding of a ARP-2 to a receptor may be accomplished by a straightforward binding inhibition/enhancement assay. According to standard techniques, the binding of a labeled (e.g., fluorescently or enzyme-labeled) antibody to ARP-2, which has been immobilized in a microtiter well, is assayed in both the presence and absence of the appropriate ligand. The change in binding is indicative of either an enhancer (increased binding) or competitive inhibitor (decreased binding) relationship between the antibody and the ligand. Such assays may be carried out in high-throughput formats (e.g., 384 well plate formats, in robotic systems) for the automated selection of monoclonal antibody candidates for use as ARP-2 ligand-binding inhibitors or enhancers.

In addition, antibodies which are useful for altering the function of ARP-2 may be assayed in functional formats, such as endothelial sprouting assays and cell migration assays described in the examples. Thus, antibodies that exhibit the appropriate anti-tumor effect may be selected without direct knowledge of a binding ligand.

### **SPARC**

Given the experiments described above, and the results of Table 1, SPARC was selected as a target for selective immuno-therapeutic agents in targeting and/or imaging brain tumors. The mature protein consists of 286 amino acids (after cleavage of the signal peptide) and contains two potential Asn-X-Thr/Ser N-glycosylation sites, located at positions 71 and 99 of the mature protein. The complete cDNA sequence encoding SPARC is provided in SEQ ID NO. 9, and the complete amino acid sequence of SPARC is provided in SEQ ID NO. 10. SPARC is an abundant 33 kDa, single chain, acidic, extracellular calcium binding protein that contains a flexible N-terminal acidic domain I (~50 amino acids), a follistatin-like (FS) domain (~75 residues), and a C-terminal extracellular calcium-binding (EC) domain with a pair of EF-hand loops (~150 residues). The N-terminal domain shows a low affinity Ca<sup>2+</sup> binding site, a transglutaminase cross linking site, and inhibits cell spreading in cell culture assays. Calcium-dependent binding of SPARC to the triple helix of several fibrillar collagen types and basement membrane collagen type IV has been mapped to the EC domain. Two isoforms have been described, bone SPARC with a molecular weight of 31,000 kDa and platelet SPARC with a molecular weight of 33,000 kDa.

As used herein, a compound that specifically binds to SPARC is any compound (such as an antibody) that has a binding affinity for any naturally occurring isoform, splice variant, or polymorphism of SPARC, explicitly including the isoforms described herein. As one of ordinary skill in the art will appreciate, such "specific" binding compounds (e.g., antibodies) may also bind to other closely related proteins that exhibit significant homology (such as greater than 90% identity, more preferably greater than 95% identity, and most preferably greater than 99% identity) with the amino acid sequence of SPARC. Such proteins include truncated forms or domains of SPARC, and recombinantly engineered alterations of SPARC. For example, a portion of SEQ ID NO. 10 may be engineered to include a non-naturally occurring cysteine for cross linking to an immunoconjugate protein, as described.

In general, it is preferred that the antibodies utilized in the compositions and methods of the invention bind to the extracellular domain (amino acids 130-280). It is preferable that this binding inhibit the activity of SPARC. The antibody may bind to the EF hand which is known to bind Ca<sup>2+</sup> with high affinity, but need not be restricted to this domain. It is to be noted that antibodies which bind to SPARC are useful in both cytotoxic and imaging embodiments of the



invention, as one of ordinary skill would expect that the concentration of SPARC in the extracellular matrix would be increased around tumor cells which over-express the protein.

When raising antibodies to SPARC the entire protein (either the unsecreted precursor or the secreted protein), or a portion thereof, may be utilized. For instance, the C terminal  
5 extracellular (EC) domain, or any portion of the flexible N-terminal domain I, or FS domain may be utilized. For instance, amino acids 125-275, which make up the EC domain, may be used. Larger SPARC proteins and domains may be produced utilizing any suitable recombinant vector/protein production system, such as the baculovirus transfection system outlined below, after being amplified from a fetal brain cDNA library (as available from, e.g., Clontech, Palo Alto, CA) or another suitable source.

When utilizing an entire protein, or a larger section of the protein, antibodies may be raised by immunizing the production animal with the protein and a suitable adjuvant (e.g., Freund's, Freund's complete, oil-in-water emulsions, etc.). In these cases, the SPARC protein (or a portion thereof) can serve as the SPARC antigen. When a smaller peptide is utilized, it is advantageous to conjugate the peptide with a larger molecule to make an immunostimulatory  
15 conjugate for use as the SPARC antigen. Commonly utilized conjugate proteins that are commercially available for such use include bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). In order to raise antibodies to particular epitopes, peptides derived from the full SPARC sequence may be utilized. Preferably, one or more 8-30 aa peptide portions of the  
20 EC domain of SPARC are utilized, with peptides in the range of 10-20 being a more economical choice. Custom-synthesized peptides in this range are available from a multitude of vendors, and can be order conjugated to KLH or BSA. Alternatively, peptides in excess of 30 amino acids may be synthesized by solid-phase methods, or may be recombinantly produced in a suitable recombinant protein production system. In order to ensure proper protein glycosylation and  
25 processing, an animal cell system (e.g., Sf9 or other insect cells, CHO or other mammalian cells) is preferred. Other information useful in designing an antigen for the production of antibodies to SPARC, including glycosylation sites, is provided in SEQ ID NO. 10.

The EC domain of human SPARC is known to interact with the collagens I, III, IV and V, and to bind to vitronectin, all of which are components of the extracellular matrix surrounding  
30 gliomas. The binding of SPARC to these molecules may play a significant role in the oncogenesis and growth of neoplastic cells in the brain. Thus, in alternative embodiments of the

compositions and methods of the invention, antibody moieties are utilized which bind to SPARC at a site on the protein that alters the binding of an extracellular molecule, such as an ECM molecule, to SPARC. Such SPARC activity altering antibodies may be utilized in therapeutic compositions in an unconjugated form (e.g., the antibody in an acceptable pharmaceutical carrier), or may be conjugated to either a therapeutic moiety (creating a double-acting therapeutic agent) or an imaging moiety (creating a dual therapeutic/imaging agent).

Selection of antibodies which alter (enhance or inhibit) the binding of a ligand to SPARC may be accomplished by a straightforward binding inhibition/enhancement assay. According to standard techniques, the binding of a labeled (e.g., fluorescently or enzyme-labeled) antibody to SPARC, which has been immobilized in a microtiter well, is assayed in both the presence and absence of the appropriate ligand. The change in binding is indicative of either an enhancer (increased binding) or competitive inhibitor (decreased binding) relationship between the antibody and the ligand. Such assays may be carried out in high-throughput formats (e.g., 384 well plate formats, in robotic systems) for the automated selection of monoclonal antibody candidates for use as SPARC ligand-binding inhibitors or enhancers.

In addition, antibodies which are useful for altering the function of SPARC may be assayed in functional formats, such as the HUVEC tube assay and cell migration assay. Thus, antibodies that exhibit the appropriate anti-tumor effect may be selected without direct knowledge of a binding ligand or molecular function.

#### **c-MET**

Given the experiments described above, and the results of Table 1, c-MET was selected as a target for selective immuno-therapeutic agents in targeting and/or imaging brain tumors. The complete cDNA sequence encoding c-MET is provided in SEQ ID NO. 11, and the complete amino acid sequence of c-MET is provided in SEQ ID NO. 12. c-MET is a type I membrane protein heterodimer. Generally, two different receptor variants originate by post-translational processing of a common single-chain precursor of 170 kDa. Isoform p190MET is formed of a 50 kDa  $\alpha$ -chain and a 145 kDa  $\beta$ -chain that are disulfide linked, and isoform p140Met is formed of a 50 kDa  $\alpha$ -chain and an 85 kDa  $\beta$ -chain, lacking the cytoplasmic kinase domain. This 85 kDa  $\beta$  chain is likely a trans-membrane glycoprotein that is bound to the cell surface. Truncated forms of c-MET containing the 50 kDa  $\alpha$ -chain and a carboxyl-terminally

truncated 75 kDa  $\beta$  sub-unit have also been described. The 75 kDa form arises by post-translational proteolytic processing, lacks the trans-membrane domain, and is secreted from the cell.

As used herein, a compound that specifically binds to c-MET is any compound (such as an antibody) that has a binding affinity for any naturally occurring isoform, splice variant, or polymorphism of c-MET, explicitly including the three isoforms described herein. As one of ordinary skill in the art will appreciate, such "specific" binding compounds (e.g., antibodies) may also bind to other closely related proteins that exhibit significant homology (such as greater than 90% identity, more preferably greater than 95% identity, and most preferably greater than 99% identity) with the amino acid sequence of c-MET. Such proteins include truncated forms or domains of c-MET, and recombinantly engineered alterations of c-MET. For example, a portion of SEQ ID NO. 12 may be engineered to include a non-naturally occurring cysteine for cross-linking to an immunoconjugate protein, as described below.

In general, it is preferred that the antibodies utilized in the compositions and methods of the invention bind to the membrane-bound isoforms of the protein, as this will more specifically target the cytotoxic therapeutic agent, or the imaging agent, to the brain tumor cell. However, embodiments that utilize antibodies that bind to the secreted isoform of the protein are also useful in the invention, as one of ordinary skill would expect that the concentration of the secreted isoform would also be increased adjacent to brain tumor cells which over-express the protein.

The amino acid sequence of full length c-MET consists of 1408 amino acids, as the sequence was first deduced by Park et al., ("Sequence of MET proto-oncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors" *Proc. nat. Acad. Sci. U.S.A.* 84:6379-6383 (1987)) and 1390 amino acids, as later deduced by Prat et al. ("C-terminal truncated forms of Met, the Hepatocyte Growth Factor" *Mol. Cell. Biol.* 11:5954-5962 (1991)). According to Prat et al., the first N-terminal amino acids 1-24 of SEQ ID NO. B' [B'] are for the most part hydrophobic, and could serve as a signal sequence for transporting the protein into the lumen of the endoplasmic reticulum. The  $\alpha$  chain makes up the extracellular domain of the mature c-MET protein and spans amino acids 24-306 of SEQ ID NO. 12. The  $\beta$  chain would consist of 1,084-5 amino acids with the predicted  $\beta$  chain extracellular domain

being amino acids 306 to 932, the single transmembrane hydrophobic segment being amino acids 933 to 955, and the intracellular domain being amino acids 956 to 1390 of SEQ ID NO 12.

When raising antibodies to c-MET, the entire protein, a dimeric subunit, or a portion thereof may be utilized. For instance, the extracellular domain of the  $\alpha$  or  $\beta$  sub-units or the secreted or extracellular portion of the truncated forms may be utilized. For instance, amino acids that constitute the  $\alpha$  sub-unit, amino acids 24-306, may be used. Larger c-MET proteins and domains may be produced utilizing any suitable recombinant vector/protein production system, such as the baculovirus transfection system outlined below, after being amplified from a fetal brain cDNA library (as available from, e.g., Clontech, Palo Alto, CA) or another suitable source.

When utilizing an entire protein, or a larger section of the protein, antibodies may be raised by immunizing the production animal with the protein and a suitable adjuvant (e.g., Freund's, Freund's complete, oil-in-water emulsions, etc.). In these cases, the c-MET protein (or a portion thereof) can serve as the c-MET antigen. When a smaller peptide is utilized, it is advantageous to conjugate the peptide with a larger molecule to make an immunostimulatory conjugate for use as the c-MET antigen. Commonly utilized conjugate proteins that are commercially available for such use include bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). In order to raise antibodies to particular epitopes, peptides derived from the full c-MET sequence may be utilized. Preferably, one or more 8-30 amino acid peptide portions of an extracellular domain of c-MET are utilized, with peptides in the range of 10-20 being a more economical choice. Custom-synthesized peptides in this range are available from a multitude of vendors, and can be order conjugated to KLH or BSA. Alternatively, peptides in excess of 30 amino acids may be synthesized by solid-phase methods, or may be recombinantly produced in a suitable recombinant protein production system. In order to ensure proper protein glycosylation and processing, an animal cell system (e.g., Sf9 or other insect cells, CHO or other mammalian cells) is preferred. Other information useful in designing an antigen for the production of antibodies to c-MET, including glycosylation sites, is provided in SEQ ID NO. 12.

The extracellular domain of human c-MET binds hepatocyte growth factor (HGF). Because HGF is largely expressed in mesenchymal and neuroectodermal tissues and released to the extracellular compartment, paracrine and/or autocrine signaling implicate tumor genesis in mesenchymal and neuroectodermal tumors and other tumor cells that over express the c-MET

receptor. Recent studies have shown that the c-MET proto-oncogene is frequently overexpressed in many types of epithelial tumors, in spontaneously transformed NIH/3T3 fibroblasts, and in peripheral nerve sheath tumors. In alternative embodiments of the compositions and methods of the invention, antibody moieties are utilized which bind to c-MET at a site on the protein which alters the binding of an extracellular ligand molecule, such as HGF, to c-MET. Such c-MET activity altering antibodies may be utilized in therapeutic compositions in an unconjugated form (e.g., the antibody in an acceptable pharmaceutical carrier), or may be conjugated to either a therapeutic moiety (creating a double-acting therapeutic agent) or an imaging moiety (creating a dual therapeutic/imaging agent).

Selection of antibodies that alter (enhance or inhibit) the binding of a ligand to c-MET may be accomplished by a straightforward binding inhibition/enhancement assay. According to standard techniques, the binding of a labeled (e.g., fluorescently or enzyme-labeled) antibody to c-MET, which has been immobilized in a microtiter well, is assayed in both the presence and absence of the ligand. The change in binding is indicative of either an enhancer (increased binding) or competitive inhibitor (decreased binding) relationship between the antibody and the ligand. Such assays may be carried out in high-throughput formats (e.g., 384 well plate formats, in robotic systems) for the automated selection of monoclonal antibody candidates for use as c-MET ligand-binding inhibitors or enhancers.

In addition, antibodies that are useful for altering the function of c-MET may be assayed in functional formats, such as the endothelial sprouting assay and cell migration assay. Thus, antibodies which exhibit the appropriate anti-tumor effect may be selected without direct knowledge of a molecular function.

#### **BEHAB**

Given the experiments described above, and the results of Table 1, BEHAB was selected as a target for selective immuno-therapeutic agents in targeting and/or imaging brain tumors. The complete cDNA sequence encoding BEHAB GPI isoform is provided in SEQ ID NO. 13, and the complete amino acid sequence of this BEHAB isoform is provided in SEQ ID NO. 14. Two isoforms have been isolated to date: a full-length isoform that is secreted into the extracellular matrix and a shorter isoform that has a hydrophobic carboxy terminus instead of the typical lectican carboxyl terminus, which predicts a glycoposphatidylinositol (GPI) anchor.

BEHAB contains an N-terminal hyaluronan (HA)-binding domain, which comprises an immunoglobulin-like loop and two proteoglycan tandem repeats, a C-terminal epidermal growth factor (EGF)-like repeat, a C-type lectin-like domain, and a complement regulatory protein (CRP)-like domain. The central region of the protein contains sites for glycosylation and proteolytic cleavage (between glu395-Ser396 of the mature protein, after signal peptide cleavage) by metallo-protease. The complete cDNA of the secreted isoform is 2878 bp encoding 912 amino acids of 99 kDa. The GPI isoform, for which sequences SEQ ID NO. 13 and SEQ ID NO. 14 are given, is 2558 bp encoding 672 amino acids of 72 kDa. The GPI-linked form is generated by a 'no splice' event, with the transcript reading through an exon/intron junction thereby extending the open reading frame to a stop codon 74 nucleotides further downstream.

As used herein, a compound that specifically binds to BEHAB is any compound (such as an antibody) that has a binding affinity for any naturally occurring isoform, splice variant, or polymorphism of BEHAB, explicitly including the two splice variants described herein. As one of ordinary skill in the art will appreciate, such "specific" binding compounds (e.g., antibodies) may also bind to other closely related proteins that exhibit significant homology (such as greater than 90% identity, more preferably greater than 95% identity, and most preferably greater than 99% identity) with the amino acid sequence of BEHAB. Such proteins include truncated forms or domains of BEHAB, and recombinantly engineered alterations of BEHAB. For example, a portion of SEQ ID NO. 14 may be engineered to include a non-naturally occurring cysteine for cross-linking to an immunoconjugate protein, as described below.

In general, it is preferred that the antibodies utilized in the compositions and methods of the invention bind to the membrane-bound isoform of the protein, as this will more specifically target the cytotoxic therapeutic agent, or the imaging agent, to the brain tumor cell. However, embodiments that utilize antibodies that bind to the secreted isoform of the protein are also useful in the invention, as one of ordinary skill would expect that the concentration of the secreted isoform would also be increased adjacent to brain tumor cells which over-express the protein.

When raising antibodies to BEHAB, the entire protein, or a portion thereof, may be utilized. For instance, any one of the aforementioned domains of the secreted protein or an extracellular portion of the truncated, membrane bound GPI form may be utilized. For instance, amino acids that constitute the hyaluronic acid binding domain, amino acids 44-247, which

includes the Ig like domain at amino acids 44-140, may be used. Larger BEHAB proteins and domains may be produced utilizing any suitable recombinant vector/protein production system, such as the baculovirus transfection system outlined below, after being amplified from a fetal brain cDNA library (as available from, e.g., Clontech, Palo alto, CA) or another suitable source.

5 When utilizing an entire protein, or a larger section of the protein, antibodies may be raised by immunizing the production animal with the protein and a suitable adjuvant (e.g., Fruend's, Fruend's complete, oil-in-water emulsions, etc.). In these cases, the Brevican protein (or a portion thereof) can serve as the BEHAB antigen. When a smaller peptide is utilized, it is advantageous to conjugate the peptide with a larger molecule to make an immunostimulatory conjugate for use as the BEHAB antigen. Commonly utilized conjugate proteins that are commercially available for such use include bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). In order to raise antibodies to particular epitopes, peptides derived from the full Brevican sequence may be utilized. Preferably, one or more 8-30 amino acid peptide portions of an extracellular domain of BEHAB are utilized, with peptides in the range of 10-20 being a more economical choice. Custom-synthesized peptides in this range are available from a multitude of vendors, and can be order conjugated to KLH or BSA. Alternatively, peptides in excess of 30 amino acids may be synthesized by solid-phase methods, or may be recombinantly produced in a suitable recombinant protein production system. In order to ensure proper protein glycosylation and processing, an animal cell system (e.g., Sf9 or other insect cells, CHO or other mammalian cells) is preferred.

15 The hyaluronic acid binding domain of human BEHAB binds to hyaluronic acid (HA). Because HA is largely expressed in the ECM surrounding gliomas and because recent studies have shown that the BEHAB protein is frequently overexpressed in primary brain tumors, it is suggested that the up-regulation of BEHAB may be a crucial step in returning the unmalleable mature extracellular matrix to a more immature matrix, permissive for cell growth, thereby promoting the progression of primary brain tumors. Thus, in alternative embodiments of the compositions and methods of the invention, antibody moieties are utilized which bind to BEHAB at a site on the protein which alters the binding of an extracellular ligand molecule (e.g., HA) to BEHAB. Such BEHAB activity altering antibodies may be utilized in therapeutic compositions in an unconjugated form (e.g., the antibody in an acceptable pharmaceutical carrier), or may be

conjugated to either a therapeutic moiety (creating a double-acting therapeutic agent) or an imaging moiety (creating a dual therapeutic/imaging agent).

Selection of antibodies that alter (enhance or inhibit) the binding of a ligand to BEHAB may be accomplished by a straightforward binding inhibition/enhancement assay. According to standard techniques, the binding of a labeled (e.g., fluorescently or enzyme-labeled) antibody to BEHAB, which has been immobilized in a microtiter well, is assayed in both the presence and absence of the ligand. The change in binding is indicative of either an enhancer (increased binding) or competitive inhibitor (decreased binding) relationship between the antibody and the ligand. Such assays may be carried out in high-throughput formats (e.g., 384 well plate formats, in robotic systems) for the automated selection of monoclonal antibody candidates for use as BEHAB ligand-binding inhibitors or enhancers.

In addition, antibodies that are useful for altering the function of BEHAB may be assayed in functional formats, such as the HUVEC tube assay and the cell migration assay described below. Thus, antibodies which exhibit the appropriate anti-tumor effect may be selected without direct knowledge of molecular function.

#### CD-44

Given the experiments described above, and the results of Table 1, CD-44 was selected as a target for selective immuno-therapeutic agents in targeting and/or imaging brain tumors. The complete cDNA sequence encoding CD-44 E is provided in SEQ ID NO. 15, and the complete amino acid sequence of CD-44, indicating various splicing variation locations, is provided in SEQ ID NO. 16. CD-44 is a proteoglycan that is expressed as two major splice variants. CD-44E is a 150 kDa protein isolated from epithelial cells. CD-44E has a C-terminal cytoplasmic tail, a hydrophobic transmembrane domain of 23 amino acids, and an N-terminal extracellular region of 248 amino acids. The extracellular domain is O-glycosylated and also binds chondroitin sulfate. In addition, CD-44E it has two of the three immunodominant epitope clusters of native gp90Hermes. CD-44E contains an additional 132 amino acids in the extracellular region. and CD-44H is a 90kDa protein isolated from hematopoietic cells. In addition, CD-44R1 and CD-44R2 are 2 isoforms expressed by hematopoietic cells. The complete cDNA sequence of the 90 kDa CD-44H isoform consist of 1795 bps, encoding a 341 amino acid protein.



As used herein, a compound that specifically binds to CD-44 is any compound (such as an antibody) that has a binding affinity for any naturally occurring isoform, splice variant, or polymorphism of CD-44, explicitly including the isoforms described herein. As one of ordinary skill in the art will appreciate, such "specific" binding compounds (e.g., antibodies) may also  
5 bind to other closely related proteins that exhibit significant homology (such as greater than 90% identity, more preferably greater than 95% identity, and most preferably greater than 99% identity) with the amino acid sequence of CD-44. Such proteins include truncated forms or domains of CD-44, and recombinantly engineered alterations of CD-44. For example, a portion of SEQ ID NO. 16 may be engineered to include a non-naturally occurring cysteine for cross-  
10 linking to an immunoconjugate protein, as described below.

According to the human full length, CD-44H protein has an overall primary structure of 90 kDa, which consist of 341 amino acids. The N-terminus is located outside of the cell and the extracellular domain consist of 248 amino acids. The C-terminus is located inside of the cell and the intracellular domain consist of 72 amino acids, while the transmembrane region consist of 21  
15 amino acids. The CD-44 gene contains 20 exons, of which exons 1-5, 15-17 and 19 encode the CD44H isoform. The intervening exons 6, 6a, 7-14 (also designated v1-v10) are alternatively spliced to generate the variant isoforms with an insertion at the membrane proximal region of the extracellular domain between amino acids 202 and 203. See Bajorath (2000). *Proteins: structure, function, and genetic*, 39:103-111; and Ilangumaram et al. *Leukemia and Lymphoma*,  
20 35:455-469.

When raising antibodies to CD-44, the entire protein, or a portion thereof, may be utilized. For instance, any portion of the extracellular domain may be utilized. For instance, the amino acids between the signal sequence and amino acid 202 may be used. Larger CD-44 proteins and domains may be produced utilizing any suitable recombinant vector/protein  
25 production system, such as the baculovirus transfection system outlined below, after being amplified from a fetal brain cDNA library (as available from, e.g., Clontech, Palo Alto, CA) or another suitable source.

When utilizing an entire protein, or a larger section of the protein, antibodies may be raised by immunizing the production animal with the protein and a suitable adjuvant (e.g.,  
30 Fruend's, Fruend's complete, oil-in-water emulsions, etc.). In these cases, the CD-44 protein (or a portion thereof) can serve as the CD-44 antigen. When a smaller peptide is utilized, it is

advantageous to conjugate the peptide with a larger molecule to make an immunostimulatory conjugate for use as the CD-44 antigen. Commonly utilized conjugate proteins that are commercially available for such use include bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). In order to raise antibodies to particular epitopes, peptides derived from the full CD-44 sequence may be utilized. Preferably, one or more 8-30 amino acid peptide portions of an extracellular domain of CD-44 are utilized, with peptides in the range of 10-20 being a more economical choice. Custom-synthesized peptides in this range are available from a multitude of vendors, and can be order conjugated to KLH or BSA. Alternatively, peptides in excess of 30 amino acids may be synthesized by solid-phase methods, or may be recombinantly produced in a suitable recombinant protein production system. In order to ensure proper protein glycosylation and processing, an animal cell system (e.g., Sf9 or other insect cells, CHO or other mammalian cells) is preferred. Other information useful in designing an antigen for the production of antibodies to CD-44, including glycosylation sites, is provided in SEQ ID NO. D'.

Hyaluronan (HA) is a polymeric glycosaminoglycan and a major component of the extracellular matrix. CD-44 is one of the principal receptors for HA. Within the normal CNS, the CD-44 protein has been localized to astrocytes in the white matter. CD-44H has been shown to be the predominant isoform in normal brain and neuroectoderm-derived tumors. Hence, the up-regulation of CD-44 may be a crucial step in brain tumor invasiveness and migration. Thus, in alternative embodiments of the compositions and methods of the invention, antibody moieties are utilized which bind to CD-44 at a site on the protein which alters the binding of an extracellular ligand molecule (e.g., HA) to CD-44. Such CD-44 activity altering antibodies may be utilized in therapeutic compositions in an unconjugated form (e.g., the antibody in an acceptable pharmaceutical carrier), or may be conjugated to either a therapeutic moiety (creating a double-acting therapeutic agent) or an imaging moiety (creating a dual therapeutic/imaging agent).

Selection of antibodies that alter (enhance or inhibit) the binding of a ligand to CD-44 may be accomplished by a straightforward binding inhibition/enhancement assay. According to standard techniques, the binding of a labeled (e.g., fluorescently or enzyme-labeled) antibody to CD-44, which has been immobilized in a microtiter well, is assayed in both the presence and absence of the ligand. The change in binding is indicative of either an enhancer (increased binding) or competitive inhibitor (decreased binding) relationship between the antibody and the

ligand. Such assays may be carried out in high-throughput formats (e.g., 384 well plate formats, in robotic systems) for the automated selection of monoclonal antibody candidates for use as CD-44 ligand-binding inhibitors or enhancers.

In addition, antibodies that are useful for altering the function of CD-44 may be assayed in functional formats, such as endothelial sprouting assay and cell migration assay. Thus, antibodies which exhibit the appropriate anti-tumor effect may be selected without direct knowledge of molecular function.

### TSPAN3

Given the experiments described above, and the results of Table 1, TSPAN3 was selected as a target for selective immuno-therapeutic agents in targeting and/or imaging brain tumors. The complete cDNA sequence encoding TSPAN3 is provided in SEQ ID NO. 17, and the complete amino acid sequence of TSPAN3 is provided in SEQ ID NO. 18. Tetraspanin is a 253 amino acid membrane bound protein. No isoforms have been isolated to date. TSPAN3, as is characteristic of the tetraspanin family, contains four transmembrane domains, putatively comprising amino acids 12-32, 51-71, 86-106, and 213-233. The protein has two putative extracellular domains, amino acids 33-50 and 107-212, and three putative cytoplasmic domains, amino acids 1-11, 72-85, and 234-235. Putative N-linked glycosylation sites are listed in SEQ ID NO. 18.

As used herein, a compound that specifically binds to TSPAN3 is any compound (such as an antibody) that has a binding affinity for any naturally occurring isoform, splice variant, or polymorphism of TSPAN3. As one of ordinary skill in the art will appreciate, such "specific" binding compounds (e.g., antibodies) may also bind to other closely related proteins that exhibit significant homology (such as greater than 90% identity, more preferably greater than 95% identity, and most preferably greater than 99% identity) with the amino acid sequence of TSPAN3. Such proteins include truncated forms or domains of TSPAN3, and recombinantly engineered alterations of TSPAN3. For example, a portion of SEQ ID NO. 18 may be engineered to include a non-naturally occurring cysteine for cross-linking to an immunoconjugate protein, as described below.

In general, it is preferred that the antibodies utilized in the compositions and methods of the invention bind to the membrane-bound isoform of the protein, as this will more specifically

target the cytotoxic therapeutic agent, or the imaging agent, to the brain tumor cell. The only currently known form of TSPAN3 is membrane-bound. However, embodiments that utilize antibodies that bind to any secreted isoform of the protein are also useful in the invention, as one of ordinary skill would expect that the concentration of the secreted isoform would also be increased adjacent to brain tumor cells which over-express the protein. Likewise, it is preferred that the antibodies utilized in the invention bind to an extracellular domain of the protein, as are described in the SEQ ID NO. 18. The cysteine residues at positions 147, 148, and 197 of SEQ ID NO. 18 in the second extracellular domain are highly conserved among the tetraspanin family and are thought to be essential for proper tetraspanin function. Thus, in some preferred embodiments of the invention, the antibodies utilized in the invention bind to an epitope comprising, or alternatively very near to, one of these cysteine residues.

When raising antibodies to TSPAN3, the entire protein, or a portion thereof, may be utilized. For instance, any one of the aforementioned domains of the secreted protein or an extracellular portion of the truncated, membrane bound GPI form may be utilized. For instance, amino acids that constitute one of the extracellular domains, amino acids 33-50 or 107-212, may be used. Larger TSPAN3 proteins and domains may be produced utilizing any suitable recombinant vector/protein production system, such as the baculovirus transfection system outlined below, after being amplified from a fetal brain cDNA library (as available from, e.g., Clontech, Palo Alto, CA) or another suitable source.

When utilizing an entire protein, or a larger section of the protein, antibodies may be raised by immunizing the production animal with the protein and a suitable adjuvant (e.g., Freund's, Freund's complete, oil-in-water emulsions, etc.). In these cases, the tetraspanin 3 protein (or a portion thereof) can serve as the TSPAN3 antigen. When a smaller peptide is utilized, it is advantageous to conjugate the peptide with a larger molecule to make an immunostimulatory conjugate for use as the TSPAN3 antigen. Commonly utilized conjugate proteins that are commercially available for such use include bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). In order to raise antibodies to particular epitopes, peptides derived from the full Brevican sequence may be utilized. Preferably, one or more 8-30 amino acid peptide portions of an extracellular domain of TSPAN3 are utilized, with peptides in the range of 10-20 being a more economical choice. Custom-synthesized peptides in this range are available from a multitude of vendors, and can be order conjugated to KLH or BSA.

Alternatively, peptides in excess of 30 amino acids may be synthesized by solid-phase methods, or may be recombinantly produced in a suitable recombinant protein production system. In order to ensure proper protein glycosylation and processing, an animal cell system (e.g., Sf9 or other insect cells, CHO or other mammalian cells) is preferred. Other information useful in designing an antigen for the production of antibodies to TSPAN3, including glycosylation sites, is provided in SEQ ID NO. 18.

In alternative embodiments of the compositions and methods of the invention, antibody moieties are utilized which bind to TSPAN3 at a site on the protein which alters the binding of an extracellular ligand molecule to TSPAN3. Such TSPAN3 activity altering antibodies may be utilized in therapeutic compositions in an unconjugated form (e.g., the antibody in an acceptable pharmaceutical carrier), or may be conjugated to either a therapeutic moiety (creating a double-acting therapeutic agent) or an imaging moiety (creating a dual therapeutic/imaging agent).

Selection of antibodies that alter (enhance or inhibit) the binding of a ligand to TSPAN3 may be accomplished by a straightforward binding inhibition/enhancement assay. According to standard techniques, the binding of a labeled (e.g., fluorescently or enzyme-labeled) antibody to TSPAN3, which has been immobilized in a microtiter well, is assayed in both the presence and absence of the ligand. The change in binding is indicative of either an enhancer (increased binding) or competitive inhibitor (decreased binding) relationship between the antibody and the ligand. Such assays may be carried out in high-throughput formats (e.g., 384 well plate formats, in robotic systems) for the automated selection of monoclonal antibody candidates for use as TSPAN3 ligand-binding inhibitors or enhancers.

In addition, antibodies that are useful for altering the function of TSPAN3 may be assayed in functional formats, such as the HUVEC tube assay and the cell migration assay described below. Thus, antibodies which exhibit the appropriate anti-tumor effect may be selected without direct knowledge of molecular function.

## **VIPR-2**

Given the experiments described above, and the results of Table 1, VIPR-2 was selected as a prime target for selective immuno-therapeutic agents in targeting and/or imaging brain tumors. The complete cDNA sequence encoding VIPR-2 is provided in SEQ ID NO. 19, and the complete amino acid sequence of VIPR-2 is provided in SEQ ID NO. 20. VIPR-2 is a seven

transmembrane spanning G-protein receptor. The complete VIPR-2 protein is encoded by 13 exons. The initiator codon of the approximated 438 amino acid-encoding open reading frame is located in exon 1 and the termination signal is located in exon 13. The 5' untranslated region extends 187 bp upstream of the initiator codon and is extremely GC-rich (80%). The polyadenylation signal is located 2416 bp downstream of the stop codon. Intron sizes range from 68 bp (intron 11) to 45 bp (intron 4), the entire human gene spans 117 kb, while the cDNA sequence spans 1317 bp. Recent studies have also isolated two VIP-2 receptor mRNAs of 4.6 kb and 2.3 kb in size.

As used herein, a compound that specifically binds to VIPR-2 is any compound (such as an antibody) that has a binding affinity for any naturally occurring isoform, splice variant, or polymorphism of VIPR-2, explicitly including any isoforms described herein. As one of ordinary skill in the art will appreciate, such "specific" binding compounds (e.g., antibodies) may also bind to other closely related proteins that exhibit significant homology (such as greater than 90% identity, more preferably greater than 95% identity, and most preferably greater than 99% identity) with the amino acid sequence of VIPR-2. Such proteins include truncated forms or domains of VIPR-2, and recombinantly engineered alterations of VIPR-2. For example, a portion of SEQ ID NO. 20 may be engineered to include a non-naturally occurring cysteine for cross-linking to an immunoconjugate protein, as described below.

In general, it is preferred that the antibodies utilized in the compositions and methods of the invention bind to the membrane-bound isoforms of the protein, as this will more specifically target the cytotoxic therapeutic agent, or the imaging agent, to the brain tumor cell. However, embodiments that utilize antibodies that bind to the secreted isoform of the protein are also useful in the invention, as one of ordinary skill would expect that the concentration of the secreted isoform would also be increased adjacent to brain tumor cells which over-express the protein.

The amino acid sequence of full length VIPR-2 consists of 437 amino acids with a predicted molecular mass is 49 kDa, as the sequence was first deduced by Lutz et al. *FEBS*. 334:3-8, 1993. Lutz et al. predicted that the receptor is a seven membrane spanning protein where in the first 22 amino acids constitute a typical hydrophobic signal sequence, and the remaining amino acids constitute two membrane spanning regions between amino acids 127 to 148 and 158 to 178, two more membrane spanning domains between amino acids 202 to 227 and

238 to 261, another between 278 to 303, and two final membrane spanning regions between 327 to 347 and 359 to 380, with three potential N-linked glycosylation sites found in the amino terminal extracellular domain at residues 57, 87 and 91. Sreedharan et al. describes the VIPR-2 receptor as being a 457 amino-acid protein encoded by a 2.8 kb cDNA of 52 kDa. Sreedharan et al. *Biochem. Biophys. Res. Commun.* 203:141-148, 1994.

When raising antibodies to VIPR-2, the entire protein or a portion thereof may be utilized. For instance, the extracellular domains of any of the seven transmembrane spanning portions of the protein may be utilized. For instance, amino acids 179 to 201 may be used. Larger VIPR-2 proteins and domains may be produced utilizing any suitable recombinant vector/protein production system, such as the baculovirus transfection system outlined below, after being amplified from a fetal brain cDNA library (as available from, e.g., Clontech, Palo Alto, CA) or another suitable source.

When utilizing an entire protein, or a larger section of the protein, antibodies may be raised by immunizing the production animal with the protein and a suitable adjuvant (e.g., Freund's, Freund's complete, oil-in-water emulsions, etc.). In these cases, the VIPR-2 protein (or a portion thereof) can serve as the VIPR-2 antigen. When a smaller peptide is utilized, it is advantageous to conjugate the peptide with a larger molecule to make an immunostimulatory conjugate for use as the VIPR-2 antigen. Commonly utilized conjugate proteins that are commercially available for such use include bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). In order to raise antibodies to particular epitopes, peptides derived from the full VIPR-2 sequence may be utilized. Preferably, one or more 8-30 amino acid peptide portions of an extracellular domain of VIPR-2 are utilized, with peptides in the range of 10-20 being a more economical choice. Custom-synthesized peptides in this range are available from a multitude of vendors, and can be order conjugated to KLH or BSA. Alternatively, peptides in excess of 30 amino acids may be synthesized by solid-phase methods, or may be recombinantly produced in a suitable recombinant protein production system. In order to ensure proper protein glycosylation and processing, an animal cell system (e.g., Sf9 or other insect cells, CHO or other mammalian cells) is preferred. Other information useful in designing an antigen for the production of antibodies to VIPR-2, including glycosylation sites, is provided in SEQ ID NO. 20.

The extracellular domain of human VIPR-2 binds PACAP-27, PACAP-38, VIP and secretin. Because these factors have been found to affect tumor cell growth, and due to the

recent discovery that the VIPR-2 receptor is overexpressed in glioblastomas (Astrocytoma grade IV), the binding of these factors to the VIPR-2 receptor may play a significant role in the oncogenesis and growth of astrocytoma cells in the brain. Thus, in alternative embodiments of the of the invention, antibody moieties are utilized which bind to VIPR-2 at a site on the protein which alters the binding of extracellular ligand molecules, such as VIP, to VIPR-2. Such VIPR-2 activity altering antibodies may be utilized in therapeutic compositions in an unconjugated form (e.g., the antibody in an acceptable pharmaceutical carrier), or may be conjugated to either a therapeutic moiety (creating a double-acting therapeutic agent) or an imaging moiety (creating a dual therapeutic/imaging agent).

Selection of antibodies that alter (enhance or inhibit) the binding of a ligand to VIPR-2 may be accomplished by a straightforward binding inhibition/enhancement assay. According to standard techniques, the binding of a labeled (e.g., fluorescently or enzyme-labeled) antibody to VIPR-2, which has been immobilized in a microtiter well, is assayed in both the presence and absence of the ligand (e.g., vasoactive intestinal peptide.) The change in binding is indicative of either an enhancer (increased binding) or competitive inhibitor (decreased binding) relationship between the antibody and the ligand. Such assays may be carried out in high-throughput formats (e.g., 384 well plate formats, in robotic systems) for the automated selection of monoclonal antibody candidates for use as VIPR-2 ligand-binding inhibitors or enhancers.

In addition, antibodies that are useful for altering the function of VIPR-2 may be assayed in functional formats, such as the HUVEC tube assay and cell migration assay. Thus, antibodies which exhibit the appropriate anti-tumor effect may be selected without direct knowledge of a binding ligand.

### PTN

Given the experiments described above, and the results of Table 1, PTN was selected as a target for selective immuno-therapeutic agents in targeting and/or imaging brain tumors. The entire PTN gene spans 65kb and 7 exons, while the mature protein is approximately 136 amino acids (after cleavage of a 32 amino acid signal peptide) with distinctive lysine and arginine-rich clusters within both N- and C-terminal domains. The complete cDNA sequence encoding PTN is provided in SEQ ID NO. 21, and the complete amino acid sequence of PTN is provided in



SEQ ID NO. 22. PTN is a 18 kDa, single chain, secreted protein with 10 conserved disulfide linked cysteine residues.

As used herein, a compound that specifically binds to PTN is any compound (such as an antibody) that has a binding affinity for any naturally occurring isoform, splice variant, or polymorphism of PTN, explicitly including the isoforms described herein. As one of ordinary skill in the art will appreciate, such "specific" binding compounds (e.g., antibodies) may also bind to other closely related proteins that exhibit significant homology (such as greater than 90% identity, more preferably greater than 95% identity, and most preferably greater than 99% identity) with the amino acid sequence of PTN. Such proteins include truncated forms or domains of PTN, and recombinantly engineered alterations of PTN. For example, a portion of SEQ ID NO. 22 may be engineered to include a non-naturally occurring cysteine for cross linking to an immunoconjugate protein, as described.

According to Milner et al. the gene sequence of PTN isolated from human genomic DNA consists of five exons and four introns. While exon 1 does not encode an amino acid sequence, exon 2 encodes the hydrophobic signal sequence of 32 amino acids, exons 3 and 4 code for the amino terminal and the ten cysteine residues, and exon 5 codes for the highly basic C-terminal domains. Interestingly, the human cDNA starts toward the end of exon 1, while the coded for protein begins at exon 2. Thus, the mature protein consist of 136 amino acids encoded by exons 2 to 5. As reported by Kretschmer et al. the minimum size of the gene is 42kb, with a mRNA of 1650 nucleotides, spanning five exons, the majority of the protein being coded for by exon 3 (174 base pairs in length) and exon 4 (162 base pairs in length). See Kretschmer et al. (1993). *Biochem. Biophys. Res. Commun.* 192:420-429.

When raising antibodies to PTN, the entire protein or a portion thereof may be utilized. For instance, amino acid domains encoded for by exons 3 and 4 (i.e. amino acids 7 to 64 or 65 to 118, respectfully). Specifically, residues 41 to 64 may be used to abolish the transformation potential of PTN. Larger PTN proteins and domains may be produced utilizing any suitable recombinant vector/protein production system, such as the baculovirus transfection system outlined below, after being amplified from a fetal brain cDNA library (as available from, e.g., Clontech, Palo Alto, CA) or another suitable source. It is to be noted that antibodies which bind to this secreted protein are useful in cytotoxic and imaging embodiments of the invention, as one

of ordinary skill would expect that the concentration of the PTN would be increased adjacent to tumor cells which over-express the protein.

When utilizing an entire protein, or a larger section of the protein, antibodies may be raised by immunizing the production animal with the protein and a suitable adjuvant (e.g., Freund's, Freund's complete, oil-in-water emulsions, etc.). In these cases, the PTN protein (or a portion thereof) can serve as the PTN antigen. When a smaller peptide is utilized, it is advantageous to conjugate the peptide with a larger molecule to make an immunostimulatory conjugate for use as the PTN antigen. Commonly utilized conjugate proteins that are commercially available for such use include bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). In order to raise antibodies to particular epitopes, peptides derived from the full PTN sequence may be utilized. Preferably, one or more 8-30 aa peptide portions of the protein are utilized, with peptides in the range of 10-20 being a more economical choice. Custom-synthesized peptides in this range are available from a multitude of vendors, and can be order conjugated to KLH or BSA. Alternatively, peptides in excess of 30 amino acids may be synthesized by solid-phase methods, or may be recombinantly produced in a suitable recombinant protein production system. In order to ensure proper protein glycosylation and processing, an animal cell system (e.g., Sf9 or other insect cells, CHO or other mammalian cells) is preferred. Other information useful in designing an antigen for the production of antibodies to PTN, including glycosylation sites, is provided in SEQ ID NO. 22.

PTN has been shown to bind to extracellular domain of RPTP beta and zeta. This binding inactivates the catalytic activity of RPTP, and PTN binds all the three major isoforms of RPTP beta and zeta. PTN has also been shown to interact with syndecan-3. Thus, in alternative embodiments of the compositions and methods of the invention, antibody moieties are utilized which bind to PTN at a site on the protein that alters the binding of a cell surface molecule, such as the ones listed above, to PTN. Such PTN activity altering antibodies may be utilized in therapeutic compositions in an unconjugated form (e.g., the antibody in an acceptable pharmaceutical carrier), or may be conjugated to either a therapeutic moiety (creating a double-acting therapeutic agent) or an imaging moiety (creating a dual therapeutic/imaging agent).

Selection of antibodies which alter (enhance or inhibit) the binding of a ligand to PTN may be accomplished by a straightforward binding inhibition/enhancement assay. According to standard techniques, the binding of a labeled (e.g., fluorescently or enzyme-labeled) antibody to

PTN, which has been immobilized in a microtiter well, is assayed in both the presence and absence of the appropriate ligand. The change in binding is indicative of either an enhancer (increased binding) or competitive inhibitor (decreased binding) relationship between the antibody and the ligand. Such assays may be carried out in high-throughput formats (e.g., 384 well plate formats, in robotic systems) for the automated selection of monoclonal antibody candidates for use as PTN ligand-binding inhibitors or enhancers.

In addition, antibodies which are useful for altering the function of PTN may be assayed in functional formats, such as the HUVEC tube assay and the cell migration assay described below. Thus, antibodies that exhibit the appropriate anti-PTN activity may be selected without direct knowledge of a binding ligand or the particular biomolecular interactions of PTN.

### **OPN**

Given the experiments described above, and the results of Table 1, OPN was selected as a target for selective immuno-therapeutic agents in targeting and/or imaging brain tumors. The mature protein consists of approximately 298 amino acids (after cleavage of a 16 amino acid signal peptide) and contains two potential Asn-Xaa-Ser N-glycosylation site, located at positions 65 and 92 of the mature protein. The complete cDNA sequence encoding OPN is provided in SEQ ID NO. 23, and the complete amino acid sequence of OPN is provided in SEQ ID NO. 24. OPN is an abundant 34 kDa, single chain, phosphorylated glycoprotein, with a presumed site for cell attachment at residues 144-148. Three isoforms have been identified to be generated by post transcriptional modification, such as alternative splicing, OPN-A, OPN-B, and OPN-C. OPN-A and OPN-B differ by the addition of 14 amino acids at residue 58 of the protein. Amino acids 58-71 are absent in OPN-B, and amino acids 31-57 are absent in OPN-C. OPN is a negatively charged, highly hydrophilic secreted protein.

As used herein, a compound that specifically binds to OPN is any compound (such as an antibody) that has a binding affinity for any naturally occurring isoform, splice variant, or polymorphism of OPN, explicitly including the three isoforms described herein. As one of ordinary skill in the art will appreciate, such "specific" binding compounds (e.g., antibodies) may also bind to other closely related proteins that exhibit significant homology (such as greater than 90% identity, more preferably greater than 95% identity, and most preferably greater than 99% identity) with the amino acid sequence of OPN. Such proteins include truncated forms or

domains of OPN, and recombinantly engineered alterations of OPN. For example, a portion of SEQ ID NO. 24 may be engineered to include a non-naturally occurring cysteine for cross linking to an immunoconjugate protein, as described.

According to Young et al. the cDNA sequence of OPN isolated from human bone cells (OPN-A) has an overall structure of approximately 34 kDA that consist of 298 amino acids, which is 14 amino acids less than the cDNA sequence of OPN isolated from human osteosarcoma by Keifer et al. (OPN-B). The cDNA transcript for OPN-A is 1.5 kb with an open reading frame of 900 nucleotides, of which the first 16 amino acids are hydrophobic in nature and probably constitute a signal sequence for the secreted protein. The OPN gene contains 7 exons that are alternatively spliced to generate the variant isoforms, the most common variant being the addition of a 42 bp (14 amino acid) sequence located at base 280 of OPN-A. See Young et al. (1990). *Genomics*, 7:491-502 and Keifer et al. *Nucleic Acids Res.* 17:3306.

When raising antibodies to OPN, the entire protein or a portion thereof may be utilized. For instance, amino acid domains 4 to 12 (from the N-terminus) or 29 to 37 (from the N-terminus) may be utilized. Larger OPN proteins and domains may be produced utilizing any suitable recombinant vector/protein production system, such as the baculovirus transfection system outlined below, after being amplified from a fetal brain cDNA library (as available from, e.g., Clontech, Palo Alto, CA) or another suitable source. It is to be noted that antibodies which bind to this secreted protein are useful in cytotoxic and imaging embodiments of the invention, as one of ordinary skill would expect that the concentration of OPN would be increased adjacent to tumor cells which over-express the protein.

When utilizing an entire protein, or a larger section of the protein, antibodies may be raised by immunizing the production animal with the protein and a suitable adjuvant (e.g., Freund's, Freund's complete, oil-in-water emulsions, etc.). In these cases, the OPN protein (or a portion thereof) can serve as the OPN antigen. When a smaller peptide is utilized, it is advantageous to conjugate the peptide with a larger molecule to make an immunostimulatory conjugate for use as the OPN antigen. Commonly utilized conjugate proteins that are commercially available for such use include bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). In order to raise antibodies to particular epitopes, peptides derived from the full OPN sequence may be utilized. Preferably, one or more 8-30 aa peptide portions of the protein are utilized, with peptides in the range of 10-20 being a more economical choice.

Custom-synthesized peptides in this range are available from a multitude of vendors, and can be order conjugated to KLH or BSA. Alternatively, peptides in excess of 30 amino acids may be synthesized by solid-phase methods, or may be recombinantly produced in a suitable recombinant protein production system. In order to ensure proper protein glycosylation and processing, an animal cell system (e.g., Sf9 or other insect cells, CHO or other mammalian cells) is preferred. Other information useful in designing an antigen for the production of antibodies to OPN, including glycosylation sites, is provided in SEQ ID NO. 22.

The cell attachment sequence of human OPN (amino acids 144 to 148) is believed to interact with various cell surface proteins (such as CD-44) to affect cell adhesion, and a highly acidic stretch composed almost exclusively of aspartic acid residues (amino acids 72 to 81) is believed to be the mineral binding site within the protein. Because CD-44 is frequently over expressed on primary brain tumors and metastases the binding of OPN to these various cell-surface adhesion protein molecules may play a significant role in the senescence and growth of tumor cells in the brain. Thus, in alternative embodiments of the compositions and methods of the invention, antibody moieties are utilized which bind to OPN at a site on the protein that alters the binding of a cell surface molecule, e.g., CD-44, to OPN. Such OPN activity altering antibodies may be utilized in therapeutic compositions in an unconjugated form (e.g., the antibody in an acceptable pharmaceutical carrier), or may be conjugated to either a therapeutic moiety (creating a double-acting therapeutic agent) or an imaging moiety (creating a dual therapeutic/imaging agent).

Selection of antibodies which alter (enhance or inhibit) the binding of a ligand to OPN may be accomplished by a straightforward binding inhibition/enhancement assay. According to standard techniques, the binding of a labeled (e.g., fluorescently or enzyme-labeled) antibody to OPN, which has been immobilized in a microtiter well, is assayed in both the presence and absence of the appropriate ligand. The change in binding is indicative of either an enhancer (increased binding) or competitive inhibitor (decreased binding) relationship between the antibody and the ligand. Such assays may be carried out in high-throughput formats (e.g., 384 well plate formats, in robotic systems) for the automated selection of monoclonal antibody candidates for use as OPN ligand-binding inhibitors or enhancers.

In addition, antibodies which are useful for altering the function of OPN may be assayed in functional formats, such as the HUVEC tube assay and the cell migration assay described

below. Thus, antibodies that exhibit the appropriate anti-OPN activity may be selected without direct knowledge of a the biomolecular role of OPN.

### **PTP $\zeta$**

PTP $\zeta$  was also selected as a prime target for selective immuno-therapeutic agents in treating or imaging brain tumors. The complete cDNA sequence encoding PTP $\zeta$  is provided in SEQ ID NO. 5, and the complete amino acid sequence of PTP $\zeta$  is provided in SEQ ID NO. 6. Three different splice variants have been described, which include two membrane bound variants (full length: PTP $\zeta$  -  $\alpha$ , and shorter version PTP $\zeta$  -  $\beta$ ) and one secreted form (Phosphacan). See Figure 1. Isoform PTP $\zeta$  -  $\alpha$  is the full length isoform, which contains the primary amino acid sequence aa 25-2314 of SEQ ID NO. 6 (aa 1-24 are a signal polypeptide). This full length long form of PTP $\zeta$  is a type I membrane protein. After the signal peptide it contains a carbonic anhydrase like (CAH) and a fibronectin type III like (FN3) domain, followed by a long cysteine free spacer (S) domain. This follows a 860 amino acid long insert domain, which can be glycosylated. After a single transmembrane segment, in the intracellular region it has 2 phosphatase domains, but only the membrane-proximal PTPase domain is catalytically active (Krueger 1992).

In Isoform PTP $\zeta$  -  $\beta$ , aa 755-1614 are missing. Isoform PTP $\zeta$  -S (phosphacan), is a secreted isoform, which is comprises the extracellular domains of PTP $\zeta$  -  $\alpha$ . Northern Blot analysis have shown that the PTP zeta is exclusively expressed in the human central nervous system. In mouse embryos, the PTP $\zeta$  transcript was mainly detected in the ventricular and subventricular zone of the brain and the spinal cord. The same pattern was detected in adult mice. Detailed studies have shown that during rat embryogenesis the two transmembrane splice variants of PTP $\zeta$  are mainly expressed in glial precursor cells and that the secretory version (Phosphacan) is more abundant in mature astrocytes which have already migrated in the ventricle zone. Applicants have characterized two additional novel splice variants, PTP $\zeta$  SM1 and PTP $\zeta$  SM2, which are described in detail below.

As used herein, a compound which specifically binds to human protein tyrosine phosphatase-zeta (PTP $\zeta$ ) is any compound (such as an antibody) which has a binding affinity for any naturally occurring isoform, splice variant, or polymorphism of PTP $\zeta$ , explicitly including

the three splice variants describe herein. For example, the compounds which specifically bind to novel isoforms PTP $\zeta$  SM1 and PTP $\zeta$  SM2, described below, are subsets of compounds which specifically bind to PTP $\zeta$ . As one of ordinary skill in the art will appreciate, such "specific" binding compounds (e.g., antibodies) may also bind to other closely related proteins which exhibit significant homology (such as greater than 90% identity, more preferably greater than 95% identity, and most preferably greater than 99% identity) with the amino acid sequence of PTP $\zeta$ . Such proteins include truncated forms or domains of PTP $\zeta$ , and recombinantly engineered alterations of PTP $\zeta$ . For example, an portion of SEQ ID NO. 6 may be engineered to include a non-naturally occurring cysteine for cross-linking to an immunoconjugate protein, as described below.

In general, it is preferred that the antibodies utilized in the compositions and methods of the invention bind to the membrane-bound isoforms of the protein, as this will more specifically target the cytotoxic therapeutic agent, or the imaging agent, to the brain tumor cell. However, embodiments which utilize antibodies which bind to the secreted isoform of the protein are also useful in the invention, as one of ordinary skill would expect that the concentration of the secreted isoform would also be increased adjacent to brain tumor cells which over-express the protein.

The amino acid sequence of full length PTP $\zeta$  consists of 2307 amino acids, as the sequence was deduced by Levy (in which aa 1722-1728 of SEQ ID NO. 2 were missing) (See also U.S. Patent Nos. 5,604,094, and 6,160,090, fully incorporated herein by reference), or 2314 amino acids as the sequence was deduced by Krueger, et al., ("A human transmembrane protein-tyrosine phosphatase, PTP zeta, is expressed in brain and has an N-terminal receptor domain homologous to carbonic anhydrases" *Proc. Nat. Acad. Sci. U.S.A.* 89:7417-7421 (1992)). Amino acids 1-24 of SEQ ID NO. 6 are a signal sequence which directs the proper placement of the transmembrane protein. The extracellular domain of the mature PTP $\zeta$  protein spans amino acids 25-1635 of SEQ ID NO. 6 in the long and secreted forms (this forms the entire secreted form), and amino acids 25-754, 1615-1635 in the short isoform. The transmembrane region of the protein spans amino acids 1636-1661 of SEQ ID NO. 6, and the balance of the protein forms the cytoplasmic domain, amino acids 1662-2314.

When raising antibodies to PTP $\zeta$ , the entire protein (any of the three isoforms) or a portion thereof may be utilized. For instance, the extracellular domain of the long or short form, the entire secreted form, or a portion of extracellular domain may be utilized. For instance, amino acids 25-754, which are common to both  $\alpha$  and  $\beta$  isoforms, may be used. Such larger

5 PTP $\zeta$  proteins and domains may be produced utilizing any suitable recombinant vector/protein production system, such as the baculovirus transfection system outlined below, after being amplified from a fetal brain cDNA library (as available from, e.g., Clontech, Palo Alto, CA) or another suitable source. When utilizing an entire protein, or a larger section of the protein, antibodies may be raised by immunizing the production animal with the protein and a suitable adjuvant (e.g., Fruend's, Fruend's complete, oil-in-water emulsions, etc.). In these cases, the

10 PTP $\zeta$  protein (or a portion thereof) can serve as the PTP $\zeta$  antigen. When a smaller peptide is utilized, it is advantageous to conjugate the peptide with a larger molecule to make an immunostimulatory conjugate for use as the PTP $\zeta$  antigen. Commonly utilized conjugate proteins which are commercially available for such use include bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). In order to raise antibodies to particular epitopes, peptides derived from the full PTP $\zeta$  sequence may be utilized. Preferably, one or more 8-30 aa peptide portions of an extracellular domain of PTP $\zeta$  are utilized, with peptides in the range of 10-20 being a more economical choice. Custom-synthesized peptides in this range are available from a multitude of vendors, and can be order conjugated to KLH or BSA. Alternatively,

20 peptides in excess of 30 amino acids may be synthesized by solid-phase methods, or may be recombinantly produced in a suitable recombinant protein production system. In order to ensure proper protein glycosylation and processing, an animal cell system (e.g., Sf9 or other insect cells, CHO or other mammalian cells) is preferred. Other information useful in designing an antigen for the production of antibodies to PTP $\zeta$ , including glycosylation sites, is provided in SEQ ID

25 NO. 6.

The extracellular domain of human PTP $\zeta$  is known to bind to tenascin-C, tenascin-R, pleiotrophin (NM\_002825), midkine (NM\_002391), FGF-2 (XM\_00366), Nr-CAM (NM\_005010), L1/Ng-CAM, contactin (NM\_001843), N-CAM (XM\_006332), and axonin-1 (NM\_005076.) The first 5 of these molecules are either components of the extracellular matrix

30 in gliomas or are soluble factors known to be present in gliomas, and the latter 4 are neuronal



surface molecules. The binding of PTP $\zeta$  to these molecules may play a significant role in the oncogenesis and growth of neoplastic cells in the brain. Thus, in alternative embodiments of the compositions and methods of the invention, antibody moieties are utilized which bind to PTP $\zeta$  at a site on the protein which alters the binding of an extracellular ligand molecule to PTP $\zeta$ . Such

5 PTP $\zeta$  activity altering antibodies may be utilized in therapeutic compositions in an unconjugated form (e.g., the antibody in an acceptable pharmaceutical carrier), or may be conjugated to either a therapeutic moiety (creating a double-acting therapeutic agent) or an imaging moiety (creating a dual therapeutic/imaging agent).

Selection of antibodies which alter (enhance or inhibit) the binding of a ligand to PTP $\zeta$  may be accomplished by a straightforward binding inhibition/enhancement assay. According to standard techniques, the binding of a labeled (e.g., fluorescently or enzyme-labeled) antibody to PTP $\zeta$ , which has been immobilized in a microtiter well, is assayed in both the presence and absence of the ligand. The change in binding is indicative of either an enhancer (increased binding) or competitive inhibitor (decreased binding) relationship between the antibody and the

10

15 ligand. Such assays may be carried out in high-throughput formats (e.g., 384 well plate formats, in robotic systems) for the automated selection of monoclonal antibody candidates for use as PTP $\zeta$  ligand-binding inhibitors or enhancers.

In addition, antibodies which are useful for altering the function of PTP $\zeta$  may be assayed in functional formats, such as the HUVEC tube assay and the cell migration assay described

20 below. Thus, antibodies that exhibit the appropriate anti-PTP $\zeta$  activity may be selected without direct knowledge of a the biomolecular role of PTP $\zeta$ .

### **Novel PTP $\zeta$ Splice Variants PTP $\zeta$ SM1 and PTP $\zeta$ SM2**

25 In addition to the known variants of PTP $\zeta$  for use in the invention, applicants have identified two novel splice variant isoforms of PTP $\zeta$ , SM1 and SM2, from their clone libraries, see Figure 2. These novel isoforms, PTP $\zeta$  SM1 and PTP $\zeta$  SM2, differ in structure from the three known isoforms heretofore disclosed, as is illustrated in Figure 3. As only cDNA sequences for the known splice variants had been previously disclosed, rather than the full gene sequence,

applicants verified the location of the novel sequences by comparison of the known splice variant sequences and the novel sequences with a publicly available genomic sequence database.

The protein PTP $\zeta$  SM1 (amino acid sequence SEQ ID NO. 2, cDNA sequence SEQ ID NO. 1) comprises the amino acids encoded by the first nine exons of PTP $\zeta$  -  $\alpha$ , with three unique additional carboxy terminal amino acids, see Figure 2. These are encoded by additional 3' mRNA sequence (nucleotides 1262-1272 of SEQ ID NO. 1) from the intron of the gene between exons nine and ten. The PTP $\zeta$  SM1 clone was isolated from a human fetal brain cDNA library, and has been shown to be expressed in several human glioblastoma cell lines. Expression of the SM1 splice variant has also been confirmed in primary brain tumor samples. The protein comprises only extracellular domains of PTP $\zeta$ , and is expected to be secreted by the cell. Thus, PTP $\zeta$  SM1 may serve a cell signaling or messenger function, and may have bind to a receptor on the surface of cells which are associated with or part of central nervous system tissues. Thus, antibodies specific for PTP $\zeta$  SM1, and not specific for the other splicing isoforms of PTP $\zeta$ , may be especially efficacious in the brain tumor therapeutic or imaging compositions of the invention. The PTP $\zeta$  SM1 protein mainly comprises the carbonic anhydrase-like domain which has been identified in PTP $\zeta$   $\alpha$ .

Applicants have explored the relationship between the putative carbonic anhydrase domain of PTP $\zeta$  SM1 (SEQ ID NO. 2) and other human carbonic anhydrase domains from carbonic anhydrase III (SEQ ID NO. 25), carbonic anhydrase I (SEQ ID NO. 26), and carbonic anhydrase VIX [e] (SEQ ID NO. 27), shown below:

		1				50
	cah3 human	~~~~~	~~~~~	~~~~~	~~~~~AKEW	GYASHNGPDH
	cah1 human	~~~~~	~~~~~	~~~~~	~~~~~ASPDW	GYDDKNGPEQ
25	cahe human	~~~~~	~~~~~ML	FSALLLEVIW	ILAADGGQHW	TYEGPHGQDH
	rptpzetaexon9_frame1	MRILKRFLAC	IQLLCVCRLD	WANGYYRQQR	KLVEEIG..W	SYTGALNQKN
		51				100
	cah3_human	WHELFPNAKG	ENQSPIELHT	KDIRHD...P	SLQPWSVSYD	GGSAKTILNN
30	cah1_human	WSKLYPIANG	NNQSPVDIKT	SETKHD...T	SLKPISVSYN	PATAKEIINV
	cahe_human	WPASYPECGN	NAQSPIDIQT	DSVTFDPDLP	ALQPHGYDQP	GTEPLDLHNN
	rptpzetaexon9_frame1	WGKKYPTCNS	PKQSPINIDE	DLTQVNVNLK	KLKFQGWDKT	SLENTFIHNT
		101				150
35	cah3_human	GKTCRVVFDD	TYDRSMLRGG	PLPGPYRLRQ	FHLHWGS.S.	DDHGSEHTVD
	cah1_human	GHSFHVNFED	NDNRSVLKGG	PFSDSYRLFQ	FHFHWGS.T.	NEHGSEHTVD
	cahe_human	GHTVQLSLP.	....STLYLG	GLPRKYVAAQ	LHLHWGQ.KG	SPGGSEHQIN
	rptpzetaexon9_frame1	GKTVEINLTN	DYRVS...GG	VSEMVFKASK	ITFHWGKCNM	SSDGSEHSLE

		151		200
	cah3_human	GVKYAAELHL	VHWN.PKYNT FKEALKQRDG	IAVIGIFLKI GH.ENGEFQI
	cah1_human	GVKYSAELHV	AHWNSAKYSS LAEAASKADG	LAVIGVLMKV GE.ANPKLQK
5	cahe_human	SEATFAELHI	VHYDSDSYDS LSEAAERPQG	LAVLGILIEV GETKNIAYEH
	rptpzetaexon9_frame1	GQKFPLEMQI	YCFDADRFSS FEEAVKGKGK	LRALSILFEV GTEENLDFKA
		201		250
	cah3_human	FLDALDKIKT	KGKEAPFTKF DPSCFLFPACR	.DYWTYQGSF TTPPCEECIV
10	cah1_human	VLDALQAIKT	KGKRAPFTNF DPSTLLPSSL	.DFWTYPGSL THPPLYESVT
	cahe_human	ILSHLHEVRH	KDQKTSVPPF NLRELLPKQL	GQYFRYNGSL TTPPCYQSVL
	rptpzetaexon9_frame1	IIDGVESVSR	FGKQAALDPF ILLNLLPNST	DKYIYINGSL TSPPCTDTVD
		251		300
15	cah3_human	WLLLKEPMTV	SSDQMAKLRS LLSSAENEPP	VP...LVSNW RPPQPINNRV
	cah1_human	WIICKESISV	SSEQLAQFRS LLSNVEGDNA	VP...MQHNN RPTQPLKGRT
	cahe_human	WTVFYRRSQI	SMEQLEKLQG TLFSTEEEPS	KL...LVQNY RALQPLNQRM
	rptpzetaexon9_frame1	WIVFKDTVSI	SESQLAVFCE VLTMQQSGYV	MLMDYLQNNF REQQYKFSRQ
		301		350
	cah3_human	VRASFK~~~~	~~~~~	~~~~~
	cah1_human	VRASF~~~~	~~~~~	~~~~~
	cahe_human	VFASFIQAGS	SYTTGEMLSL GVGILVGCLC	LLLAVYFIAR KIRKKRLNR
20	rptpzetaexon9_frame1	VFSSYTGKEE	IHEAVCSSEP ENVQADPENY	TSLLVTWERP RVVYDTMIEK
		351		380
	cah3_human	~~~~~	~~~~~	~~~~~
	cah1_human	~~~~~	~~~~~	~~~~~
	cahe_human	KSVVFTSAQA	TTEA~~~~~	~~~~~
25	rptpzetaexon9_frame1	FAVLYQQLDG	EDQTKHEFLT DGYQDLVTI*	
		30		

Based on alignment with these catalytically active carbonic anhydrases, it seems unlikely that the CA domain could function as a carbonic anhydrase enzyme. Two of the three histidines implicated in binding of the catalytic zinc are missing from the CA domain of the receptor. In active enzymes there is a conserved HxHWG{18,20}ELH motif (the three histidines bind zinc), however, in the receptor this is modified to TFHWG{18,20}EMQ; i.e. two of the three critical zinc atoms would be missing. For comparison, it has been found that a carbonic anhydrase related protein (CAH 8) that lacks just one of these histidines also lacks catalytic activity.

The protein PTP $\zeta$  SM2 (amino acid sequence SEQ ID NO. 4) comprises the amino acids encoded by all exons of PTP $\zeta$  -  $\alpha$ , plus a 116 nucleotide "extra" exon, in the correct reading frame, between exons 23 and 24 (nucleotides 6229-6345 of SEQ ID NO. 3). This extra exon, designated exon 23a, contains a portion of the intron sequence between exons 23 and 24 of the PTP $\zeta$  gene. PTP $\zeta$  SM2 expression has been verified in several human glioblastoma cell lines, and has also been confirmed in primary brain tumor samples. As PTP $\zeta$  SM2 comprises all the domains of PTP $\zeta$   $\alpha$ , the protein is expected to be membrane-bound. The extra exon lies within

the cytoplasmic domain of the protein, and thus may alter the protein tyrosine phosphatase function of PTP $\zeta$  SM2.

A novel splicing variant PTP $\zeta$  protein having an amino acid sequence which includes the amino acid sequence of PTP $\zeta$  SM1 (SEQ ID NO. 2) or PTP $\zeta$  SM2 (SEQ. ID NO. 4) may be produced by recombinant techniques known in the art utilizing any suitable vector, in any suitable host cell. The term "vector" is intended to include any physical or biochemical vehicle containing nucleic acid polymers of interest, by which those nucleic acid polymers are transferred into a host cell, thereby transfecting that cell with the introduced nucleic acid polymers. The transfected nucleic acid sequence preferably contains a control sequence, such as a promoter sequence, suitable for transcription of the nucleic acid sequence in the host cell. Examples of vectors include DNA plasmids, viruses, liposomes, particle gun pellets, and transfection vectors known to those of skill in the molecular biology arts. The term "host cell" is intended to mean the target cell for vector transformation, in which the transferred nucleic acid polymer will be replicated and/or expressed. Although bacterial cells may be suitable for production of the proteins for antibody production or structural study purposes, eukaryotic cell hosts are preferred for production of the protein for functional assays or therapeutic purposes. Preferred eukaryotic cell hosts include insect cell lines (e.g, Sf9, Sf21, or High Five™ cell lines), and mammalian cell lines (e.g., HeLa, CHO-K1, COS-7, COS-1, HEK293, HEPG2, Jurkat, MDCK, PAE, PC-12, and other acceptable mammalian cell lines). Thus, the invention also provides vectors incorporating a nucleic acid sequence encoding PTP $\zeta$  SM1 or PTP $\zeta$  SM2, as well as host cells which express the proteins.

It is common in the molecular biology arts to utilize additional functional amino acid domains or proteins fused with a protein sequence of interest for purification or detection purposes. Such additional functionalities include, for example, polyhistidine domains, c-myc domains (specifically comprising amino acids 410-419 of the human c-myc oncogene product),  $\beta$ -galactosidase,  $\beta$ -glucuronidase, glutathione-S-transferase, maltose binding protein, human influenza virus hemagglutinin, green fluorescent protein, chloramphenicol acetyltransferase, luciferase, thioredoxin, and others. After purification (e.g., by antibody-affinity chromatography) or detection, these extra amino acid sequences may be cleaved (e.g., by thrombin, enterokinase, Factor Xa, or other protease) to yield a functional mature protein. Thus,

the PTP $\zeta$  SM1 and SM2 proteins of the invention also encompass proteins comprising the amino acid sequence of SEQ ID NO. 2 or SEQ ID NO. 4 and such additional amino acid functionalities.

The invention also provides polypeptides which have a unique activity of PTP $\zeta$  SM1 or PTP $\zeta$  SM2 which is not shared by the other PTP $\zeta$  splice variant (e.g., an antigenic epitope) and which include a portion of the amino acid sequence of PTP $\zeta$  SM1 or PTP $\zeta$  SM2 which is at least about 8 to 12 amino acid residues in length, more preferably at least about 20 amino acids in length. These polypeptides preferably comprise an amino acid sequence which is not found in PTP $\zeta$   $\alpha$ , PTP $\zeta$   $\beta$ , or phosphacan, wherein the included portion of the sequence confers the unique activity on the polypeptide. Such polypeptides may be utilized as described above to produce affinity reagents which specifically bind to PTP $\zeta$  splice variants SM1 or SM2, but do not bind to the other known splice variants of PTP $\zeta$ . The invention thus provides such specific affinity reagents, which may be produced from such polypeptides, or from an entire PTP $\zeta$  SM1 or PTP $\zeta$  SM2 protein. In preferred embodiments these affinity reagents are antibodies or antibody fragments.

In addition, although the understanding of the field of protein biochemistry is not as complete as that of molecular genetics, the person of ordinary skill in the art of biochemistry is capable of predicting, with reasonable certainty, when certain substitutions to the primary amino acid sequence structure of a protein will not result in any appreciable modification of a protein's structure or function. Such conservative substitutions are made by replacing an amino acid in the sequence with another containing a side chain with like charge, size, and other characteristics. Conservative substitutions in a protein sequence which would be expected to have minimal to no impact on protein structure or function can be readily devised by a person of ordinary skill in the biochemical arts. To the extent that such conservative substitutions can be made while retaining 90%, preferably 95%, and more preferably 99% or more identity to SEQ. ID NO. 2 or SEQ ID NO. 4, and maintain the activity of the native PTP $\zeta$  SM1 or PTP $\zeta$  SM2 protein, such altered proteins are within the scope of the present invention.

The invention also provides nucleic acid polymers encoding the PTP $\zeta$  splice variants SM1 or SM2. These nucleic acid polymers most preferably comprises a nucleic acid sequence of SEQ. ID NO. 1 or SEQ ID NO. 3, or the predictable variants thereof which one of ordinary skill of the art could derive using the degeneracy of the genetic code. Such nucleic acid polymers are

useful for the production of PTP $\zeta$  SM1 or PTP $\zeta$  SM2 by recombinant methods, as described above.

The invention also encompasses nucleic acid probes or primers which hybridize to the mRNA encoding PTP $\zeta$  splice variants SM1 or SM2, but not mRNA encoding other known splice variants of PTP $\zeta$ . Such probes or primers provided by the invention are preferably able to hybridize with SEQ. ID NO. 1 or SEQ. ID NO. 3 (or their complements) under stringent conditions (e.g., 0.5X to 2X SSC buffer, 0.1% SDS, and a temperature of 55-65° C), but do not hybridize to SEQ ID NO. 5 (or its complement) under the same conditions. These PTP $\zeta$  SM1 or PTP $\zeta$  SM2 coding sequence specific probes are preferably from about 16 to about 40 nucleotides in length, more preferably from about 18 nucleotides to about 30 nucleotides in length. However, probes may be of a smaller size, preferably from about 8 to about 15 nucleotides in length, if two or more probes are hybridized to adjacent sequences, so that terminal nucleic acid base-stacking interactions may stabilize their hybridization. In preferred embodiments of PTP $\zeta$  SM1 specific nucleic acid probes, the probes hybridize at or near the novel splice site at the 3' end of exon 9, or its complement. In preferred embodiments of PTP $\zeta$  SM2 specific probes, the probes hybridize at or adjacent to a location selected from: the novel splice site at the 3' end of exon 23, at least a portion of the novel exon 23a, the novel splice site at the 5' end of exon 24, or the complement of any one of these.

Because PTP $\zeta$  SM1 and PTP $\zeta$  SM2 have been shown to be expressed in glioblastoma cell lines and primary tumors, the level of the expression of these splice variants may be useful for staging or characterizing glioblastoma cells. Such cells may be extracted, for instance, from a primary tumor. Thus, the invention provides for the monitoring of the relative expression level of PTP $\zeta$  SM1 or PTP $\zeta$  SM2, or both, in relation to each other or to one or more of the known PTP $\zeta$  splice variants. In one preferred embodiment, the level of expression of PTP $\zeta$  SM1 is compare to at least one other splice variant selected from PTP $\zeta$  SM2, PTP $\zeta$   $\alpha$ , PTP $\zeta$   $\beta$ , and phosphacan. In another preferred embodiment, the level of expression of PTP $\zeta$  SM2 is compare to at least one other splice variant selected from PTP $\zeta$  SM1, PTP $\zeta$   $\alpha$ , PTP $\zeta$   $\beta$ , and phosphacan. Such comparison may be made in either a qualitative or quantitative manner. One means for comparison is by hybridizing splice-variant specific nucleic acid probes to a sample of nucleic acids (which may be amplified) obtained from brain tumor cells. Alternatively, the expression

level of the splice variants may be deduced by the amplification of splice variant nucleic acid sequences, and the analysis of the size of those amplified products using methods known in the art. In another alternative embodiment, protein levels may be studied utilizing splice-variant specific antibodies in either sandwich immunoassay or in-situ staining formats. Various expression level assay techniques are known to those of skill in the molecular biological arts, and thus the specific techniques mentioned above should be considered merely exemplary.

#### Antibodies for use in the antibody-therapeutics methods of the invention

Generally, as the term is utilized in the specification, "antibody" or "antibody moiety" is intended to include any polypeptide chain-containing molecular structure that has a specific shape which fits to and recognizes an epitope, where one or more non-covalent binding interactions stabilize the complex between the molecular structure and the epitope. Antibodies which bind specifically to one of the brain tumor protein targets are referred to as anti-brain tumor protein target antibodies, or  $\alpha(T_{BT})$ , or more specifically  $\alpha(ARP2)$ ,  $\alpha(SPARC)$ ,  $\alpha(CMET)$ ,  $\alpha(CD44)$ ,  $\alpha(BEHAB)$ ,  $\alpha(TSPAN3)$ ,  $\alpha(VIPR2)$ ,  $\alpha(OPN)$ ,  $\alpha(PTN)$ , and  $\alpha(PTP\zeta)$ . The specific or selective fit of a given structure and its specific epitope is sometimes referred to as a "lock and key" fit. The archetypal antibody molecule is the immunoglobulin, and all types of immunoglobulins (IgG, IgM, IgA, IgE, IgD, etc.), from all sources (e.g., human, rodent, rabbit, cow, sheep, pig, dog, other mammal, chicken, turkey, emu, other avians, etc.) are considered to be "antibodies." Antibodies utilized in the present invention may be polyclonal antibodies, although monoclonal antibodies are preferred because they may be reproduced by cell culture or recombinantly, and may be modified to reduce their antigenicity.

Polyclonal antibodies may be raised by a standard protocol by injecting a production animal with an antigenic composition, formulated as described above. See, e.g., Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988. In one such technique, an  $T_{BT}$  antigen comprising an antigenic portion of the brain tumor protein targets' polypeptide is initially injected into any of a wide variety of mammals (e.g., mice, rats, rabbits, sheep or goats). Alternatively, in order to generate antibodies to relatively short peptide portions of the brain tumor protein target (see discussion above), a superior immune response may be elicited if the polypeptide is joined to a carrier protein, such as ovalbumin, BSA or KLH. The peptide-conjugate is injected into the animal host, preferably according to a predetermined

schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the polypeptide may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

5 Alternatively, for monoclonal antibodies, hybridomas may be formed by isolating the stimulated immune cells, such as those from the spleen of the inoculated animal. These cells are then fused to immortalized cells, such as myeloma cells or transformed cells, which are capable of replicating indefinitely in cell culture, thereby producing an immortal, immunoglobulin-secreting cell line. The immortal cell line utilized is preferably selected to be deficient in enzymes necessary for the utilization of certain nutrients. Many such cell lines (such as myelomas) are known to those skilled in the art, and include, for example: thymidine kinase (TK) or hypoxanthine-guanine phosphoriboxyl transferase (HGPRT). These deficiencies allow selection for fused cells according to their ability to grow on, for example, hypoxanthine aminopterin-thymidine medium (HAT).

10  
15 Preferably, the immortal fusion partners utilized are derived from a line that does not secrete immunoglobulin. The resulting fused cells, or hybridomas, are cultured under conditions that allow for the survival of fused, but not unfused, cells and the resulting colonies screened for the production of the desired monoclonal antibodies. Colonies producing such antibodies are cloned, expanded, and grown so as to produce large quantities of antibody, see Kohler and  
20 Milstein, 1975 Nature 256:495 (the disclosures of which are hereby incorporated by reference).

Large quantities of monoclonal antibodies from the secreting hybridomas may then be produced by injecting the clones into the peritoneal cavity of mice and harvesting the ascites fluid therefrom. The mice, preferably primed with pristine, or some other tumor-promoter, and immunosuppressed chemically or by irradiation, may be any of various suitable strains known to  
25 those in the art. The ascites fluid is harvested from the mice and the monoclonal antibody purified therefrom, for example, by CM Sepharose column or other chromatographic means. Alternatively, the hybridomas may be cultured in vitro or as suspension cultures. Batch, continuous culture, or other suitable culture processes may be utilized. Monoclonal antibodies are then recovered from the culture medium or supernatant.

30 Several monoclonal antibodies against various isoforms of the brain tumor protein targets are currently available from commercial sources. For instance, a non-exclusive list of available



commercial antibodies includes: for SPARC / Osteonectin, from *Zymed*, mouse anti-bovine MAb (cross-reactivity with human), suitable for ELISA, WB, IH (paraffin), Cat# 33-5500; for c-MET, from *Zymed*, rabbit anti-human polyclonal, suitable for ELISA, WB, IH. Cat# 71-8000, and from *RDI*, rabbit anti-human MAb, suitable for WB, IP, IH. Cat# RDI-MET Cabr.; for CD44, from *RDI*, mouse anti-human MAb, only for IH and FACS, Cat# RDI-M1676clb., and from *Lab vision*, mouse anti-human MAb, known to block binding of hyaluronic acid to its receptor CD44, "CD44/H-CAM Ab-2"; for Brevican/BEHAB, from *BD Transduction Lab.*, a mouse anti-human MAb, WB, IF, Cat# B68820; for VIP 2 receptor, from *Exalpha*, mouse anti-rat (possible human cross-specificity, which is easily assayed) MAb, WB, IH. Cat#2140M; for Laminin receptor 67 kDa, from *Lab vision*, mouse anti-human MAb, IH, ELISA, not for WB. "laminin receptor Ab-1"; for Osteopontin, from *Chemicon*, rat anti-human MAb, raised against rh-Osteopontin – recognizes native protein well, WB, IH, ELISA. "MAB3057"; for Pleiotrophin, from *R&D* goat anti-human polyclonal, WB, recognizes rh-Pleiotrophin. "BAF252", and from *Oncogene* goat anti-human polyclonal, WB, ELISA, detects rh-Pleiotrophin. "PC187L".; for PTP $\zeta$  -  $\alpha$  and PTP $\zeta$  -  $\beta$ , from *BD Transduction Labs*, mouse anti-human MAb (WB, IH, IF), denominated "R20720" and from *Chemicon*, mouse anti-human MAb (WB, IH, IP), denominated "MAB5210", which recognizes both of the transmembrane isoforms, and also recognizes the soluble isoform (phosphacan, PTP $\zeta$ -S). These antibodies are suitable for use in the compositions of the present invention, especially in Fab fragment form (which eliminates significant portions of the antigenic mouse constant heavy and light chain regions). However, it is preferred that such antibodies be humanized or chimerized according to one of the procedures outlined below.

In addition, the antibodies or antigen binding fragments may be produced by genetic engineering. In this technique, as with the standard hybridoma procedure, antibody-producing cells are sensitized to the desired antigen or immunogen. The messenger RNA isolated from the immune spleen cells or hybridomas is used as a template to make cDNA using PCR amplification. A library of vectors, each containing one heavy chain gene and one light chain gene retaining the initial antigen specificity, is produced by insertion of appropriate sections of the amplified immunoglobulin cDNA into the expression vectors. A combinatorial library is constructed by combining the heavy chain gene library with the light chain gene library. This results in a library of clones which co-express a heavy and light chain (resembling the Fab

fragment or antigen binding fragment of an antibody molecule). The vectors that carry these genes are co-transfected into a host (e.g. bacteria, insect cells, mammalian cells, or other suitable protein production host cell.). When antibody gene synthesis is induced in the transfected host, the heavy and light chain proteins self-assemble to produce active antibodies that can be detected by screening with the antigen or immunogen.

Preferably, recombinant antibodies are produced in a recombinant protein production system which correctly glycosylates and processes the immunoglobulin chains, such as insect or mammalian cells. An advantage to using insect cells which utilize recombinant baculoviruses for the production of antibodies for use in the present invention is that the baculovirus system allows production of mutant antibodies much more rapidly than stably transfected mammalian cell lines. In addition, insect cells have been shown to correctly process and glycosylate eukaryotic proteins, which prokaryotic cells do not. Finally, the baculovirus expression of foreign protein has been shown to constitute as much as 50-75% of the total cellular protein late in viral infection, making this system an excellent means of producing milligram quantities of the recombinant antibodies.

The use of the baculovirus *Autographia californica* nuclear polyhedrosis virus (AcNPV) and recombinant viral stocks in *Spodoptera frugiperda* (Sf9) cells to prepare large quantities of protein has been described by Smith et al. (1985), Summers and Smith (1987). A preferred method of preparing recombinant antibodies is through the expression of DNA encoding recombinant antibody (produced by screening, as above, or by protein engineering to include more human-like domains, as discussed below) via the baculoviral expression system in Sf9 insect cells. Production of recombinant proteins in Sf9 cells is well known in the art, and one of ordinary skill would be able to select from a number of acceptable protocols (e.g., that described in U.S. Patent No. 6,603,905).

It should be noted that antibodies which have a reduced propensity to induce a violent or detrimental immune response in humans (such as anaphylactic shock), and which also exhibit a reduced propensity for priming an immune response which would prevent repeated dosage with the antibody therapeutic or imaging agent (e.g., the human-anti-murine-antibody "HAMA" response), are preferred for use in the invention. These antibodies are preferred for all administrative routes, including intrathecal administration. Even though the brain is relatively isolated in the cranial cavity, behind the blood brain barrier, an immune response still can occur

in the form of increased leukocyte infiltration, and inflammation. Although some increased immune response against the tumor is desirable, the concurrent binding and inactivation of the therapeutic or imaging agent generally outweighs this benefit. Thus, humanized, chimeric, or xenogenic human antibodies, which produce less of an immune response when administered to humans, are preferred for use in the present invention.

Chimeric antibodies may be made by recombinant means by combining the murine variable light and heavy chain regions (VK and VH), obtained from a murine (or other animal-derived) hybridoma clone, with the human constant light and heavy chain regions, in order to produce an antibody with predominantly human domains. The production of such chimeric antibodies is well known in the art, and may be achieved by standard means (as described, e.g., in U.S. Patent No. 5,624,659, incorporated fully herein by reference). Humanized antibodies are engineered to contain even more human-like immunoglobulin domains, and incorporate only the complementarity-determining regions of the animal-derived antibody. This is accomplished by carefully examining the sequence of the hyper-variable loops of the variable regions of the monoclonal antibody, and fitting them to the structure of the human antibody chains. Although facially complex, the process is straightforward in practice. See, e.g., U.S. Patent No. 6,187,287, incorporated fully herein by reference.

Alternatively, polyclonal or monoclonal antibodies may be produced from animals which have been genetically altered to produce human immunoglobulins, such as the Abgenix XenoMouse or the Medarex HuMAb® technology. The transgenic animal may be produced by initially producing a "knock-out" animal which does not produce the animal's natural antibodies, and stably transforming the animal with a human antibody locus (e.g., by the use of a human artificial chromosome). Only human antibodies are then made by the animal. Techniques for generating such animals, and deriving antibodies therefrom, are described in U.S. Patents No. 6,162,963 and 6,150,584, incorporated fully herein by reference. Such fully human xenogenic antibodies are a preferred antibody for use in the methods and compositions of the present invention.

Alternatively, single chain antibodies (Fv, as described below) can be produced from phage libraries containing human variable regions. See U.S. Patent No. 6,174,708, incorporated fully herein by reference. Also see Kuan, C. T., Reist, C. J., Foulon, C. F., Lorimer, I. A., Archer, G., Pegram, C. N., Pastan, I., Zalutsky, M. R., and Bigner, D. D. (1999). 125I-labeled anti-

epidermal growth factor receptor-viii single-chain Fv exhibits specific and high-level targeting of glioma xenografts. *Clin Cancer Res.* 5, 1539-49; Lorimer, I. A., Keppler-Hafkemeyer, A., Beers, R. A., Pegram, C. N., Bigner, D. D., and Pastan, I. (1996). Recombinant immunotoxins specific for a mutant epidermal growth factor receptor: targeting with a single chain antibody variable domain isolated by phage display. *Proc. Nat. Acad. Sci. U S A* 93, 14815-20; Pastan, I. H., Archer, G. E., McLendon, R. E., Friedman, H. S., Fuchs, H. E., Wang, Q. C., Pai, L. H., Herndon, J., and Bigner, D. D. (1995). Intrathecal administration of single-chain immunotoxin, LMB-7 [B3(Fv)- PE38], produces cures of carcinomatous meningitis in a rat model. *Proc Natl. Acad. Sci U S A* 92, 2765-9, all of which are incorporated by reference fully herein.

In addition to entire immunoglobulins (or their recombinant counterparts), immunoglobulin fragments comprising the epitope binding site (e.g., Fab', F(ab')<sub>2</sub>, or other fragments) are useful as antibody moieties in the present invention. Such antibody fragments may be generated from whole immunoglobulins by ficin, pepsin, papain, or other protease cleavage. "Fragment," or minimal immunoglobulins may be designed utilizing recombinant immunoglobulin techniques. For instance "Fv" immunoglobulins for use in the present invention may be produced by linking a variable light chain region to a variable heavy chain region via a peptide linker (e.g., poly-glycine or another sequence which does not form an alpha helix or beta sheet motif).

Fv fragments are heterodimers of the variable heavy chain domain (V<sub>H</sub>) and the variable light chain domain (V<sub>L</sub>). The heterodimers of heavy and light chain domains that occur in whole IgG, for example, are connected by a disulfide bond. Recombinant Fvs in which V<sub>H</sub> and V<sub>L</sub> are connected by a peptide linker are typically stable, see, for example, Huston et al., *Proc. Natl. Acad. Sci. USA* 85:5879-5883 (1988) and Bird et al., *Science* 242:423-426 (1988), both fully incorporated herein, by reference. These are single chain Fvs which have been found to retain specificity and affinity and have been shown to be useful for imaging tumors and to make recombinant immunotoxins for tumor therapy. However, researchers have found that some of the single chain Fvs have a reduced affinity for antigen and the peptide linker can interfere with binding. Improved Fv's have been also been made which comprise stabilizing disulfide bonds between the V<sub>H</sub> and V<sub>L</sub> regions, as described in U.S. Patent No. 6,147,203, incorporated fully herein by reference. Any of these minimal antibodies may be utilized in the present invention,

and those which are humanized to avoid HAMA reactions are preferred for use in embodiments of the invention.

In addition, derivatized immunoglobulins with added chemical linkers, detectable moieties [fluorescent dyes, enzymes, substrates, chemiluminescent moieties], or specific binding moieties [such as streptavidin, avidin, or biotin] may be utilized in the methods and compositions of the present invention. For convenience, the term "antibody" or "antibody moiety" will be used throughout to generally refer to molecules which specifically bind to an epitope of the brain tumor protein targets, although the term will encompass all immunoglobulins, derivatives, fragments, recombinant or engineered immunoglobulins, and modified immunoglobulins, as described above.

Candidate anti-T<sub>BT</sub> antibodies can be tested for anti-T<sub>BT</sub> activity by any suitable standard means. As a first screen, the antibodies may be tested for binding against the brain tumor protein target antigen utilized to produce them, or against the entire brain tumor protein target extracellular domain or protein. As a second screen, anti-T<sub>BT</sub> candidates may be tested for binding to an appropriate glioblastoma cell line (i.e., one which approximates primary tumor brain tumor protein target expression), or to primary tumor tissue samples. For these screens, the anti-T<sub>BT</sub> candidate antibody may be labeled for detection (e.g., with fluorescein or another fluorescent moiety, or with an enzyme such as horseradish peroxidase). After selective binding to the brain tumor protein target is established, the candidate antibody, or an antibody conjugate produced as described below, may be tested for appropriate activity (i.e., the ability to decrease tumor cell growth and/or to aid in visualizing tumor cells) in an *in vivo* model, such as an appropriate glioblastoma cell line, or in a mouse or rat human brain tumor model, as described below.

#### General Functional Assay Methods for Antibodies for Use in the Invention

In addition to the specific binding assays and protein- specific functional assays described for individual proteins above, antibodies which are useful for altering the function of ARP-2, SPARC, c-MET, BEHAB, CD-44, TSPN3, PTN, OPN, VIPR-2, or PTP $\zeta$  may be assayed in functional formats, such as glioblastoma cell culture or mouse/rat CNS tumor model studies. In glioblastoma cell models of activity, expression of the protein is first verified in the particular cell strain to be used. If necessary, the cell line may be stably transfected with a coding sequence

of the protein under the control of an appropriate constituent promoter, in order to express the protein at a level comparable to that found in primary tumors. The ability of the glioblastoma cells to survive in the presence of the candidate function-altering anti-protein antibody is then determined. In addition to cell-survival assays, cell migration assays, as described below in Example 1, may be utilized to determine the effect of the candidate antibody therapeutic agent on the tumor-like behavior of the cells. Alternatively, if the brain tumor protein target is involved in angiogenesis, or endothelial cell sprouting assays such as described in Example 2 may be utilized to determine the ability of the candidate antibody therapeutic to inhibit vascular neogenesis, an important function in tumor biology.

Similarly, *in vivo* models for human brain tumors, particularly nude mice/SCID mice model or rat models, have been described [Antunes, L., Angioi-Duprez, K. S., Bracard, S. R., Klein-Monhoven, N. A., Le Faou, A. E., Duprez, A. M., and Plenat, F. M. (2000). Analysis of tissue chimerism in nude mouse brain and abdominal xenograft models of human glioblastoma multiforme: what does it tell us about the models and about glioblastoma biology and therapy? *J Histochem Cytochem* 48, 847-58; Price, A., Shi, Q., Morris, D., Wilcox, M. E., Brasher, P. M., Rewcastle, N. B., Shalinsky, D., Zou, H., Appelt, K., Johnston, R. N., Yong, V. W., Edwards, D., and Forsyth, P. (1999). Marked inhibition of tumor growth in a malignant glioma tumor model by a novel synthetic matrix metalloproteinase inhibitor AG3340. *Clin Cancer Res* 5, 845-54; and Senner, V., Sturm, A., Hoess, N., Wassmann, H., and Paulus, W. (2000). *In vivo* glioma model enabling regulated gene expression. *Acta Neuropathol* (Berl) 99, 603-8.] Once correct expression of the protein in the tumor model is verified, the effect of the candidate anti-protein antibodies on the tumor masses in these models can be evaluated, wherein the ability of the anti-protein antibody candidates to alter protein activity is indicated by a decrease in tumor growth or a reduction in the tumor mass. Thus, antibodies that exhibit the appropriate anti-tumor effect may be selected without direct knowledge of the particular biomolecular role of the protein in oncogenesis.

#### Therapeutic and Imaging Moieties, and Methods for Conjugating them with anti-PTP $\zeta$ Antibodies to Use in the Compositions and Methods of the Invention

As described above, the anti-T<sub>BT</sub> antibodies for use in the present invention may have utility without conjugation when the native activity of the brain tumor protein target is altered in

the tumor cell. Such antibodies, which may be selected as described above, may be utilized without further modification to include a cytotoxic or imaging moiety. These types of compositions have the advantage of reduced toxicity (in that only the toxicity of the antibody moieties themselves must be taken into account when dosing), and are simpler to manufacture.

5 Thus, non-conjugated activity altering anti-T<sub>BT</sub> antibody therapeutics are a preferred embodiment of the invention. However, the conjugation of cytotoxic or imaging agents is yet another preferred embodiment when utilizing these antibodies because the added moieties add functionality to the therapeutic.

Thus, in many preferred embodiments of the invention, the anti-T<sub>BT</sub> antibodies may be coupled or conjugated to one or more therapeutic cytotoxic or imaging moieties. As used herein, "cytotoxic moiety" (C) simply means a moiety which inhibits cell growth or promotes cell death when proximate to or absorbed by the cell. Suitable cytotoxic moieties in this regard include radioactive isotopes (radionuclides), chemotoxic agents such as differentiation inducers and small chemotoxic drugs, toxin proteins, and derivatives thereof. As utilized herein, "imaging moiety" (I) means a moiety which can be utilized to increase contrast between a tumor and the surrounding healthy tissue in a visualization technique (e.g., radiography, positron-emission tomography, magnetic resonance imaging, direct or indirect visual inspection). Thus, suitable imaging moieties include radiography moieties (e.g. heavy metals and radiation emitting moieties), positron emitting moieties, magnetic resonance contrast moieties, and optically visible moieties (e.g., fluorescent or visible-spectrum dyes, visible particles, etc.). It will be appreciated by one of ordinary skill that some overlap exists between what is a therapeutic moiety and what is an imaging moiety. For instance <sup>212</sup>Pb and <sup>212</sup>Bi are both useful radioisotopes for therapeutic compositions, but are also electron-dense, and thus provide contrast for X-ray radiographic imaging techniques, and can also be utilized in scintillation imaging techniques.

25 In general, therapeutic or imaging agents may be conjugated to the anti-PTP $\zeta$  moiety by any suitable technique, with appropriate consideration of the need for pharmacokinetic stability and reduced overall toxicity to the patient. A therapeutic agent may be coupled to a suitable antibody moiety either directly or indirectly (e.g. via a linker group). A direct reaction between an agent and an antibody is possible when each possesses a functional group capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulfhydryl group, may be capable of reacting with a carbonyl-containing group, such as an anhydride or an acid halide, or with an

alkyl group containing a good leaving group (e.g., a halide). Alternatively, a suitable chemical linker group may be used. A linker group can function as a spacer to distance an antibody from an agent in order to avoid interference with binding capabilities. A linker group can also serve to increase the chemical reactivity of a substituent on a moiety or an antibody, and thus increase the coupling efficiency. An increase in chemical reactivity may also facilitate the use of moieties, or functional groups on moieties, which otherwise would not be possible.

Suitable linkage chemistries include maleimidyl linkers and alkyl halide linkers (which react with a sulfhydryl on the antibody moiety) and succinimidyl linkers (which react with a primary amine on the antibody moiety). Several primary amine and sulfhydryl groups are present on immunoglobulins, and additional groups may be designed into recombinant immunoglobulin molecules. It will be evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional (such as those described in the catalog of the Pierce Chemical Co., Rockford, Ill.), may be employed as a linker group. Coupling may be effected, for example, through amino groups, carboxyl groups, sulfhydryl groups or oxidized carbohydrate residues. There are numerous references describing such methodology, e.g., U.S. Patent No. 4,671,958. As an alternative coupling method, cytotoxic or imaging moieties may be coupled to the anti-T<sub>BT</sub> antibody moiety through a an oxidized carbohydrate group at a glycosylation site, as described in U.S. Patents No. 5,057,313 and 5,156,840. Yet another alternative method of coupling the antibody moiety to the cytotoxic or imaging moiety is by the use of a non-covalent binding pair, such as streptavidin/biotin, or avidin/biotin. In these embodiments, one member of the pair is covalently coupled to the antibody moiety and the other member of the binding pair is covalently coupled to the cytotoxic or imaging moiety.

Where a cytotoxic moiety is more potent when free from the antibody portion of the immunoconjugates of the present invention, it may be desirable to use a linker group which is cleavable during or upon internalization into a cell, or which is gradually cleavable over time in the extracellular environment. A number of different cleavable linker groups have been described. The mechanisms for the intracellular release of a cytotoxic moiety agent from these linker groups include cleavage by reduction of a disulfide bond (e.g., U.S. Patent No. 4,489,710), by irradiation of a photolabile bond (e.g., U.S. Patent No. 4,625,014), by hydrolysis of derivatized amino acid side chains (e.g., U.S. Patent No. 4,638,045), by serum complement-



mediated hydrolysis (e.g., U.S. Patent No. 4,671,958), and acid-catalyzed hydrolysis (e.g., U.S. Patent No. 4,569,789).

It may be desirable to couple more than one cytotoxic and/or imaging moiety to an antibody. By poly-derivatizing the anti-T<sub>BT</sub> antibody, several cytotoxic strategies may be simultaneously implemented, an antibody may be made useful as a contrasting agent for several visualization techniques, or a therapeutic antibody may be labeled for tracking by a visualization technique. In one embodiment, multiple molecules of an imaging or cytotoxic moiety are coupled to one antibody molecule. In another embodiment, more than one type of moiety may be coupled to one antibody. Regardless of the particular embodiment, immunoconjugates with more than one moiety may be prepared in a variety of ways. For example, more than one moiety may be coupled directly to an antibody molecule, or linkers which provide multiple sites for attachment (e.g., dendrimers) can be used. Alternatively, a carrier with the capacity to hold more than one cytotoxic or imaging moiety can be used.

A carrier may bear the agents in a variety of ways, including covalent bonding either directly or via a linker group, and non-covalent associations. Suitable covalent-bond carriers include proteins such as albumins (e.g., U.S. Patent No. 4,507,234), peptides, and polysaccharides such as aminodextran (e.g., U.S. Patent No. 4,699,784), each of which have multiple sites for the attachment of moieties. A carrier may also bear an agent by non-covalent associations, such as non-covalent bonding or by encapsulation, such as within a liposome vesicle (e.g., U.S. Patents Nos. 4,429,008 and 4,873,088). Encapsulation carriers are especially useful for imaging moiety conjugation to anti-T<sub>BT</sub> antibody moieties for use in the invention, as a sufficient amount of the imaging moiety (dye, magnetic resonance contrast reagent, etc.) for detection may be more easily associated with the antibody moiety. In addition, encapsulation carriers are also useful in chemotoxic therapeutic embodiments, as they can allow the therapeutic compositions to gradually release a chemotoxic moiety over time while concentrating it in the vicinity of the tumor cells.

Carriers and linkers specific for radionuclide agents (both for use as cytotoxic moieties or positron-emission imaging moieties) include radiohalogenated small molecules and chelating compounds. For example, U.S. Patent No. 4,735,792 discloses representative radiohalogenated small molecules and their synthesis. A radionuclide chelate may be formed from chelating compounds that include those containing nitrogen and sulfur atoms as the donor atoms for

binding the metal, or metal oxide, radionuclide. For example, U.S. Patent No. 4,673,562, to Davison et al. discloses representative chelating compounds and their synthesis. Such chelation carriers are also useful for magnetic spin contrast ions for use in magnetic resonance imaging tumor visualization methods, and for the chelation of heavy metal ions for use in radiographic visualization methods.

Preferred radionuclides for use as cytotoxic moieties are radionuclides which are suitable for pharmacological administration. Such radionuclides include  $^{123}\text{I}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{90}\text{Y}$ ,  $^{211}\text{At}$ ,  $^{67}\text{Cu}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{212}\text{Pb}$ , and  $^{212}\text{Bi}$ . Iodine and astatine isotopes are more preferred radionuclides for use in the therapeutic compositions of the present invention, as a large body of literature has been accumulated regarding their use.  $^{131}\text{I}$  is particularly preferred, as are other  $\beta$ -radiation emitting nuclides, which have an effective range of several millimeters.  $^{123}\text{I}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ , or  $^{211}\text{At}$  may be conjugated to antibody moieties for use in the compositions and methods utilizing any of several known conjugation reagents, including Iodogen, *N*-succinimidyl 3- $^{211}\text{At}$ astatobenzoate, *N*-succinimidyl 3- $^{131}\text{I}$ iodobenzoate (SIB), and , *N*-succinimidyl 5- $^{131}\text{I}$ iodob-3-pyridinecarboxylate (SIPC). Any iodine isotope may be utilized in the recited iodo-reagents. For example, a suitable antibody for use in the present invention may be easily made by coupling an Fab fragment of the BD Transduction Labs R20720 anti-PTP $\zeta$  MAb with  $^{131}\text{I}$  Iodogen according to the manufacturer's instructions. Other radionuclides may be conjugated to anti-T<sub>BT</sub> antibody moieties by suitable chelation agents known to those of skill in the nuclear medicine arts.

Preferred chemotoxic agents include small-molecule drugs such as carboplatin, cisplatin, vincristine, taxanes such as paclitaxel and docetaxel, hydroxyurea, gemcitabine, vinorelbine, irinotecan, tirapazamine, matrilysin, methotrexate, pyrimidine and purine analogs, and other suitable small toxins known in the art. Preferred chemotoxin differentiation inducers include phorbol esters and butyric acid. Chemotoxic moieties may be directly conjugated to the anti-T<sub>BT</sub> antibody moiety via a chemical linker, or may encapsulated in a carrier, which is in turn coupled to the anti-T<sub>BT</sub> antibody moiety.

Preferred toxin proteins for use as cytotoxic moieties include ricins A and B, abrin, diphtheria toxin, bryodin 1 and 2, momordin, trichokirin, cholera toxin, gelonin, *Pseudomonas* exotoxin, *Shigella* toxin, pokeweed antiviral protein, and other toxin proteins known in the medicinal biochemistry arts. As these toxin agents may elicit undesirable immune responses in

the patient, especially if injected intravascularly, it is preferred that they be encapsulated in a carrier for coupling to the anti-T<sub>BT</sub> antibody moiety.

Preferred radiographic moieties for use as imaging moieties in the present invention include compounds and chelates with relatively large atoms, such as gold, iridium, technetium, barium, thallium, iodine, and their isotopes. It is preferred that less toxic radiographic imaging moieties, such as iodine or iodine isotopes, be utilized in the compositions and methods of the invention. Examples of such compositions which may be utilized for x-ray radiography are described in U.S. Patent No. 5,709,846, incorporated fully herein by reference. Such moieties may be conjugated to the anti-T<sub>BT</sub> antibody moiety through an acceptable chemical linker or chelation carrier. In addition, radionuclides which emit radiation capable of penetrating the skull may be useful for scintillation imaging techniques. Suitable radionuclides for conjugation include <sup>99</sup>Tc, <sup>111</sup>In, and <sup>67</sup>Ga. Positron emitting moieties for use in the present invention include <sup>18</sup>F, which can be easily conjugated by a fluorination reaction with the anti-T<sub>BT</sub> antibody moiety according to the method described in U.S. Patent No. 6,187,284.

Preferred magnetic resonance contrast moieties include chelates of chromium(III), manganese(II), iron(II), nickel(II), copper(II), praseodymium(III), neodymium(III), samarium(III) and ytterbium(III) ion. Because of their very strong magnetic moment, the gadolinium(III), terbium(III), dysprosium(III), holmium(III), erbium(III), and iron(III) ions are especially preferred. Examples of such chelates, suitable for magnetic resonance spin imaging, are described in U.S. Patent No. 5,733,522, incorporated fully herein by reference. Nuclear spin contrast chelates may be conjugated to the anti-T<sub>BT</sub> antibody moieties through a suitable chemical linker.

Optically visible moieties for use as imaging moieties include fluorescent dyes, or visible-spectrum dyes, visible particles, and other visible labeling moieties. Fluorescent dyes such as fluorescein, coumarin, rhodamine, bodipy Texas red, and cyanine dyes, are useful when sufficient excitation energy can be provided to the site to be inspected visually. Endoscopic visualization procedures may be more compatible with the use of such labels. For many procedures where imaging agents are useful, such as during an operation to resect a brain tumor, visible spectrum dyes are preferred. Acceptable dyes include FDA-approved food dyes and colors, which are non-toxic, although pharmaceutically acceptable dyes which have been approved for internal administration are preferred. In preferred embodiments, such dyes are

encapsulated in carrier moieties, which are in turn conjugated to the anti-T<sub>BT</sub> antibody. Alternatively, visible particles, such as colloidal gold particles or latex particles, may be coupled to the anti-T<sub>BT</sub> antibody moiety via a suitable chemical linker.

5 Delivery of Therapeutic and Imaging Agents to the Patient:

The Blood Brain Barrier (BBB) and Administration Strategies:

At one time, the BBB was not considered to present a problem in the diagnosis and treatment of brain tumors, because early scans of human brain tumors suggested that the BTB (blood tumor barrier) was "leaky." However, as the size of the molecule increases, the rate of movement across the barrier decreases. The BBB has been demonstrated to be heterogeneous in experimental human tumor xenograft animal models and in human patients. This lack of uniformity is because of the reduced integrity of tight junctions in the capillary endothelial cells of the tumor neovasculature, intratumoral variation in permeability, and altered intratumoral blood flow (*Fuchs et al, 1990, Cancer research 50, 1954-59, Groothuis et al., 1984, Prog.Exp.Tumor Res.*) Thus, although the BBB may not pose a delivery problem for some tumors in some patients, this cannot be said for all brain tumors. In addition, a preferred mode of administration of the therapeutics of the invention is after removal of the main tumor mass (resection of the tumor), which destroys much of the "leaky" neovasculature. Moreover, as brain carcinomas are usually pervasive throughout the organ, therapies which are directed towards eradicating all tumor-producing cells cannot rely exclusively on the localized tumor neovasculature.

A first strategy for drug delivery through the BBB entails disruption of the BBB, either by osmotic means such as mannitol or leukotrienes, or biochemically by the use of vasoactive substances such as bradykinin. The potential for using BBB opening to target specific agents to brain tumors is also an option. In preferred embodiments, a BBB disrupting agent is co-administered with the therapeutic or imaging compositions of the invention when the compositions are administered by intravascular injection. Other strategies to go through the BBB may entail the use of endogenous transport systems, including carrier-mediated transporters such as glucose and amino acid carriers, receptor-mediated transcytosis for insulin or transferrin, and active efflux transporters such as p-glycoprotein. Active transport moieties may also be

conjugated to the therapeutic or imaging compounds for use in the invention to facilitate transport across the epithelial wall of the blood vessel. However, the best current strategy for drug delivery behind the BBB is by intrathecal delivery of therapeutics or imaging agents directly to the cranium, as through an Ommaya reservoir.

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Delivery/Administration of Therapeutic Antibodies:

For administration, the antibody-therapeutic or antibody-imaging agent will generally be mixed, prior to administration, with a non-toxic, pharmaceutically acceptable carrier substance. Usually, this will be an aqueous solution, such as normal saline or phosphate-buffered saline (PBS), Ringer's solution, lactate-Ringer's solution, or any isotonic physiologically acceptable solution for administration by the chosen means. Preferably, the solution is sterile and pyrogen-free, and is manufactured and packaged under current Good Manufacturing Processes (GMPs), as approved by the FDA. The clinician of ordinary skill is familiar with appropriate ranges for pH, tonicity, and additives or preservatives when formulating pharmaceutical compositions for administration by intravascular injection, intrathecal injection, injection into the cerebro-spinal fluid, direct injection into the tumor, or by other routes. In addition to additives for adjusting pH or tonicity, the antibody-therapeutics and antibody-imaging agents may be stabilized against aggregation and polymerization with amino acids and non-ionic detergents, polysorbate, and polyethylene glycol. Optionally, additional stabilizers may include various physiologically-acceptable carbohydrates and salts. Also, polyvinylpyrrolidone may be added in addition to the amino acid. Suitable therapeutic immunoglobulin solutions which are stabilized for storage and administration to humans are described in U.S. Patent No. 5,945,098, incorporated fully herein by reference. Other agents, such as human serum albumin (HSA), may be added to the therapeutic or imaging composition to stabilize the antibody conjugates.

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The compositions of the invention may be administered using any medically appropriate procedure, e.g., intravascular (intravenous, intraarterial, intracapillary) administration, injection into the cerebrospinal fluid, intracavity or direct injection in the tumor. Intrathecal administration may be carried out through the use of an Ommaya reservoir, in accordance with known techniques. (F. Balis et al., Am J. Pediatr. Hematol. Oncol. 11, 74, 76 (1989)). For the imaging compositions of the invention, administration via intravascular injection is preferred for pre-operative visualization of the tumor. Post-operative visualization or visualization concurrent

with an operation may be through intrathecal or intracavity administration, as through an Ommaya reservoir, or also by intravascular administration.

Intravascular injection may be by intravenous or intraarterial injection: carotid artery injection is thought to assist in administration to the brain, and is thus preferred. Antibody-agents injected into the blood stream have been shown to cross the blood-brain barrier and to infiltrate the cranial cavity to some extent, usually in the range of  $10^{-4}$  to  $10^{-3}\%$  [?UNITS?] injected dose per gram. This rate of uptake may be sufficient for imaging reagents, and also may be useful for tumor cell specific cytotoxic agents (e.g, those specifically directed to the inhibition of the function of tumor-cell overexpressed proteins). However, in order to achieve therapeutic concentrations of the antibody-therapeutic agents without unacceptable toxicity to the patient, it is preferred that the therapeutics compositions be administered by intrathecal injection, direct injection, or injection into the cerebro-spinal fluid.

Thus, a preferred method for administration of the therapeutic compositions of the invention is by depositing it into the inner cavity of a cystic tumor by any suitable technique, such as by direct injection (aided by stereotaxic positioning of an injection syringe, if necessary) or by placing the tip of an Ommaya reservoir into a cavity, or cyst, for administration. Where the tumor is a solid tumor, the antibody may be administered by first creating a resection cavity in the location of the tumor. This procedure differs from an ordinary craniotomy and tumor resection only in a few minor respects. As tumor resection is a common treatment procedure, and is often indicated to relieve pressure, administration of the therapeutic compositions of the invention following tumor resection is a preferred embodiment of the treatment methods of the invention. Following gross total resection in a standard neurosurgical fashion, the cavity is preferable rinsed with saline until all bleeding is stopped by cauterization. Next the pia-arachnoid membrane, surrounding the tumor cavity at the surface, is cauterized to enhance the formation of fibroblastic reaction and scarring in the pia-arachnoid area. The result is the formation of an enclosed, fluid-filled cavity within the brain tissue at the location from where the tumor was removed. After the cyst has been formed, either the tip of an Ommaya reservoir or a micro catheter, which is connected to a pump device and allows the continuous infusion of an antibody solution into the cavity, can be placed into the cavity. See, e.g., U.S. Patent No. 5,558,852, incorporated fully herein by reference.

Alternatively, a convection-enhanced delivery catheter may be implanted directly into the tumor mass, into a natural or surgically created cyst, or into the normal brain mass. Such convection-enhanced pharmaceutical composition delivery devices greatly improve the diffusion of the composition throughout the brain mass. The implanted catheters of these delivery devices  
5 utilize high-flow microinfusion (with flow rates in the range of about 0.5 to 15.0  $\mu\text{l}/\text{minute}$ ), rather than diffusive flow, to deliver the therapeutic or imaging composition to the brain and/or tumor mass. Such devices are described in U.S. Patent No. 5,720,720, incorporated fully herein by reference.

10 The effective amount of the therapeutic antibody-conjugate composition or of the imaging antibody-conjugate compositions to be given to a particular patient will depend on a variety of factors, several of which will be different from patient to patient. A competent clinician will be able to determine an effective amount of a therapeutic antibody-conjugate composition to administer to a patient to retard the growth and promote the death of tumor cells, or an effective amount of an imaging composition to administer to a patient to facilitate the  
15 visualization of a tumor. Dosage of the antibody-conjugate will depend on the treatment of the tumor, route of administration, the nature of the therapeutics, sensitivity of the tumor to the therapeutics, etc. Utilizing  $\text{LD}_{50}$  animal data, and other information available for the conjugated cytotoxic or imaging moiety, a clinician can determine the maximum safe dose for an individual, depending on the route of administration. For instance, an intravenously administered dose may  
20 be more than an intrathecally administered dose, given the greater body of fluid into which the therapeutic composition is being administered. Similarly, compositions which are rapidly cleared from the body may be administered at higher doses, or in repeated doses, in order to maintain a therapeutic concentration. Imaging moieties are typically less toxic than cytotoxic moieties and may be administered in higher doses in some embodiments. Utilizing ordinary  
25 skill, the competent clinician will be able to optimize the dosage of a particular therapeutic or imaging composition in the course of routine clinical trials.

Typically the dosage will be 0.001 to 100 milligrams of conjugate per kilogram subject body weight. Doses in the range of 0.01 to 1 mg per kilogram of patient body weight may be utilized for a radionuclide therapeutic composition which is administered intrathecally.

30 Relatively large doses, in the range of 0.1 to 10 mg per kilogram of patient body weight, may be used for imaging conjugates with a relatively non-toxic imaging moiety. The amount utilized

will depend on the sensitivity of the imaging method, and the relative toxicity of the imaging moiety. In a therapeutic example, where the therapeutic composition comprises a  $^{131}\text{I}$  cytotoxic moiety, the dosage to the patient will typically start at a lower range of 10 mCi, and go up to 100, 300 or even 500 mCi. Stated otherwise, where the therapeutic agent is  $^{131}\text{I}$ , the dosage to the patient will typically be from 5,000 Rads to 100,000 Rads (preferably at least 13,000 Rads, or even at least 50,000 Rads). Doses for other radionuclides are typically selected so that the tumoricidal dose will be equivalent to the foregoing range for  $^{131}\text{I}$ . Similarly, chemotoxic or toxin protein doses may be scaled accordingly.

The antibody conjugate can be administered to the subject in a series of more than one administration. For therapeutic compositions, regular periodic administration (e.g., every 2-3 days) will sometimes be required, or may be desirable to reduce toxicity. For therapeutic compositions which will be utilized in repeated-dose regimens, antibody moieties which do not provoke HAMA or other immune responses are preferred. The imaging antibody conjugate compositions may be administered at an appropriate time before the visualization technique. For example, administration within an hour before direct visual inspection may be appropriate, or administration within twelve hours before an MRI scan may be appropriate. Care should be taken, however, to not allow too much time to pass between administration and visualization, as the imaging compound may eventually be cleared from the patient's system.

In addition to the use of imaging antibody conjugates for simple visualization, these compositions may be utilized as a "dry run" for more toxic cytotoxic antibody conjugates. If the same antibody moiety is utilized for the imaging conjugate as for the therapeutic conjugate, the physician may first use a visualization technique to determine precisely where in the brain the cytotoxic conjugate will concentrate. If a sufficient degree of tissue selectivity is not achieved (e.g., if the tumor cells are too dispersed in the normal tissue, or if the particular brain tumor protein target chosen is not sufficiently overexpressed in the particular patient's tumor cells), then the physician may choose another brain tumor protein target. The provision of numerous brain tumor protein targets by the present invention, along with both imaging and therapeutic agents, allows a high degree of flexibility in designing an effective treatment regimen for the individual patient.

#### Combination Therapies of the Invention



As mentioned previously, brain tumors tend to be heterogeneous in character, and pervasive throughout the brain tissue. This combination often makes them difficult to treat, as individual portions of the tumor cells in any particular patient may have differing biological characteristic. Thus, in some cases, it may be preferred to use various combinations of therapeutic or imaging agents, as described above in the Summary of Invention, in order to more fully target all of the cells exhibiting tumorigenic characteristics. Such combination treatments may be by administering blended antibody therapeutic or imaging compositions, individually prepared as described above, and administering the blended therapeutic to the patient as described. The skilled administering physician will be able to take such factors as combined toxicity, and individual antibody agent efficacy, into account when administering such combined agents. Additionally, those of skill in the art will be able to screen the antibodies to avoid potential cross-reaction with each other, in order to assure full efficacy of each antibody therapeutic or imaging agent.

Alternatively, several individual brain tumor protein target compositions may be administered simultaneously or in succession for a combined therapy. This may be desirable to avoid accumulated toxicity from several antibody conjugate reagents, or to more closely monitor potential adverse reactions to the individual antibody reagents. Thus, cycles such as where a first antibody therapeutic agent is administered on day one, followed by a second on day two, then a period with out administration, followed by re-administration of the antibody therapeutics on different successive days, is comprehended within the present invention.

## EXAMPLES

### **Example 1: Identification of Two New Splicing Variant Isoforms of PTP $\zeta$ : PTP $\zeta$ SM1 and SM2**

The mRNA nucleotide sequence for PTP $\zeta$  SM1 was identified in a human fetal brain phage cDNA library by sequencing.

The mRNA nucleotide sequence for PTP $\zeta$  SM2 was identified by PCR amplification of adult human brain cDNA, and sequencing of the resulting nucleic acids.

For the RT-PCR analyses performed below, total RNA was isolated from either cells (glioblastoma cultured lines) or tissue using Trizole (Gibco Life Technologies, Inc.), following the manufacture's protocol. cDNA was generated from total RNA using the 1<sup>st</sup> Strand synthesis kit from Gibco Life Technologies, Inc., and an oligo dT<sub>30</sub> anchored primer. For each RT-PCR  
5 reaction, 1 µl of cDNA was utilized. The PCR reaction was carried out using an Advantage 2 kit (Clontech) under standard conditions. The products of the PCR reactions were confirmed via sequencing.

Both clones were verified by RT-PCR analysis of glioblastoma cell lines and primary tumors. For PTP $\zeta$  SM1, primers CAGCAGTTGGATGGAAGAGGAC [SEQ ID NO. 28] and CACTGAGATTCTGGCACTATTC [SEQ ID NO. 29] were used, producing an identifiable  
10 1116 bp product. RT-PCR analysis was performed, confirming expression of the SM1 splice variant in 11 of 17 different glioblastoma cell lines tested, fetal brain, and adult brain, using the unique 3' end and portion of the 3' untranslated region as the hybridization target for the probe. In addition, RT-PCR analysis was performed on 28 primary brain tumor samples, confirming  
15 expression of the PTP $\zeta$  SM1 variant in 16 of the 28 tumors.

For PTP $\zeta$  SM2, primers AACAAATCCAGGGTCTCACTC [SEQ ID NO. 30] and TTGACTGGCTCAGGAGTATAG [SEQ ID NO. 31] were used, which produce a 130 bp product when the extra exon 23a is present, and a no product when the exon 23a is absent. RT-PCR analysis was performed, confirming expression in 6 of 17 different glioblastoma cell lines  
20 tested. In addition, RT-PCR analysis was performed on 28 primary brain tumor samples, confirming expression of the PTP $\zeta$  SM1 variant in 19 of the 28 tumors.

For comparison, RT-PCR analysis was also done for the expression of PTP $\zeta$  -  $\alpha$  (primers CTGATAATGAGGGCTCCCAAC [SEQ ID NO. 32] and CTCTGCACTTCCTGGTAAACTCT [SEQ ID NO. 33]) and PTP $\zeta$  -  $\beta$  (primers  
25 CAGCAGTTGGATGGAAGAGGAC [SEQ ID NO. 34] and CTCTGCACTTCCTGGTAAACTCT [SEQ ID NO. 35]) in the 28 brain tumor tissue samples. PTP $\zeta$  -  $\alpha$  was shown to be expressed in 16 of the 28 samples, and the short form PTP $\zeta$  -  $\beta$  was shown to be expressed in 19 of the 28 samples.

The nucleotide sequence alignment of the two new splice variants with the reference  
30 sequence for PTP $\zeta$  -  $\alpha$  is shown in the following table:

**TABLE 2**

PTP 5'	PTP 3'	PAC 1 5'	PAC 1 3'	Corresponding Exon Key:				
1	48	87274	87321	5' UTR	PAC 1: RP5-1062J16			
					BAC: RP11-384A20			
70	205	87343	87487	1	PAC 2: RP5-1049N15			
205	272	142076	142143	2				
		<b>BAC 5'</b>	<b>BAC 3'</b>					
291	451	24001	24161	*	3	* 88 nt deletion seen in 5' PCR clone from PTP 363-451		
450	603	28570	28723		4			
602	701	32814	32888		5			
698	772	32814	32888		6			
766	924	39695	39853		7			
922	1075	39995	40148		8			
1074	1261	52411	52598	*	9	* not spliced at 1261 in phage library clones		
1260	1387	53910	54037		10			
1387	1435	60644	60692		11			
1432	2346	66362	67276		5' 12 (end of BAC)			
		<b>PAC 2 5'</b>	<b>PAC 2 3'</b>					
2147	4409	1	2263		mid 12			
4437	4987	2294	2844		3' 12			
4925	5133	8027	8224		13			
5131	5224	17505	17598		14			
5223	5310	20427	20514		15			
5309	5332	23048	23071		16			
5329	5428	23234	23333		17			
5429	5512	25555	25638		18			
5512	5646	27710	27844		19			
5572	5602	42925	42955	*	Duplicate of mid 19	* duplicated regions of exons 19		
5646	5768	28408	28530		most of 20 (-12 bp 3')	and 26 vary by one aa / two nt		
5791	5945	29770	29934		21 (-10 bp 5')			
5943	6082	31560	31699		22			
6080	6228	33375	33523	~	23	~116 nt insert seen b/w exons 23 & 24 in 3' PCR clone: maps to PAC b/w 23 &		
24								
6225	6322	40379	40476	~	24	<b>PTP location</b>	<b>PAC 2 5'</b>	<b>PAC 2 3'</b>
6322	6397	40820	40895		25	6228	36744	36629
6396	6526	42864	42994		26			
6457	6487	27770	27800	*	Duplicate of mid 26			
6525	6673	43895	44043		27			
6671	6816	47753	47898		28			
6816	6952	48708	48844		29			

\*\*BOUNDARIES DETERMINED FROM HOMO SAPIENS CHROMOSOME 7 WORKING DRAFT (NT\_007845.3)\*\*

Nucleotide location refers to position in full length RPTPZ (accession M93426)

## Example 2: Cell Migration Assay For Determining Antibody Activity on Protein Targets

Tumor cells are known to migrate more rapidly towards chemoattractants. The cell migration assay measures the ability of a cell to migrate. The ability to migrate is taken as a measure of tumorigenicity. Chemoattractants generally used are fetal bovine serum, pleiotrophin, bFGF, and VEGF. Thus, this assay can be used to determine migration capability of a cell in which the gene has been knocked down or the gene of interest is being overexpressed.

The ChemoTx<sup>®</sup> disposable chemotaxis system (Neuroprobe, Inc., Gaithersburg, MD) is used according to the manufacturer's instructions, with a few modifications. Briefly, glioblastoma cultured cells from cell line G55T2 are prepared by splitting the cells the day

before the assay is performed. A ChemoTx<sup>®</sup> chamber with the following specifications is used: Pore size 8  $\mu\text{m}$ , exposed filter area 8  $\text{mm}^2$ , exposed filter area diameter 3.2 mm. The plate configuration is: 30  $\mu\text{l}$  per well, 96 well plate. The membrane type is: Track-etched polycarbonate.

5 In preparation for the assays, the filter membrane is coated in 100ml PBS containing 0.1% acetic acid and 3.5 ml Vitrogen 100 (from Cohesion) at 37°C overnight. About 30 minutes before starting the assay the coated membrane is washed and rinsed with PBS containing 0.1% BSA. Cells are harvested by using the standard technique (trypsin-EDTA). The cells are washed once with DMEM 10% FBS, and then spun at 1000 RPM, for 5 minutes at room  
10 temperature. The pellet is resuspended in DMEM without serum, containing 0.1% BSA (serum free medium). The cells are spun and resuspended again in serum free medium, and then spun and resuspended in the amount of serum free medium needed to provide a concentration of 1 mio. cells / ml, or 25,000 cells per 25ul. Just prior to the assay, a suitable amount of the antibody to be tested for anti-target function activity is added to the cell suspension.

15 For the assay, a standard chemoattractant is used to measure the mobility of the cells. The chemoattractants are diluted in serum free medium. A suitable unspecific chemoattractant is DMEM with 5% FBS. The chemoattractant solutions and control solutions without chemoattractant are pipetted (29  $\mu\text{l}$ ) into the lower plate wells. After placing and securing the filter plate over the lower wells, ensuring contact with the solution in the bottom wells, serial  
20 dilutions of the cell suspension are pipetted onto each site on the filter top. The plates are then covered and incubated at 37° C, 5% CO<sub>2</sub>, for 3-4 hours.

25 After incubation, the upper filter side is rinsed with PBS and exposed upper filter areas are cleaned with wet cotton swabs. The filter is stained using Diff-Quik<sup>™</sup> (VWR) dye kit, according to the manufacturer's instructions. The migrated cells are counted on the lower filter side using a microscope (Magnification 200x), by counting of 5 high power field sections per well.

**Example 2: HUVEC(Human umbilical vein endothelial cells)**  
**Endothelial Sprouting assay For Determining**  
**Antibody Activity on Protein Targets**

Cell-sprouting morphology can be utilized as an easily visualized assay to determine the inhibitory effect of a candidate antibody on the protein target function for protein targets which stimulate endothelial cell sprouting, such as ARP2. Such assays have been described extensively in the literature (Nehls, V., et al., Histochem. Cell Biol. 104: 459-466 (1995); Koblizek, T. I., et al., Curr. Biol. 8: 529-532 (1988); and Kwak, H.J., et al., FEBS Lett. 448: 249-253). Briefly, a endothelial cells from a suitable source, such as HUVECs or PPAECs (porcine pulmonary artery endothelial cells) are grown to confluence on microcarrier (MC) beads (diameter 175  $\mu$ m, available from Sigma) and placed into a 2.5 mg/ml fibrinogen gel containing the protein target at an appropriate effective concentration (200ng/ml is an suitable starting concentration, which the skilled practitioner may optimize) and the antibody in an appropriate range of concentrations (this will depend on antibody titer and affinity for the target), and 200 units/ml Trasylol (available from Bayer). Fibrin gels are incubated in M-199 with a daily supplement of the same amount of recombinant protein and antibody, 2.0% heat-inactivated fetal bovine serum, and 200 units/ml Trasylol. After three days, the extent of sprouting is determined using a phase-contrast microscope (such as those available from Zeiss). A decrease in cell sprouting as compared to controls without antibody indicates a reduction in protein target activity by the antibody.

The foregoing is intended to be illustrative of the embodiments of the present invention, and are not intended to limit the invention in any way. Although the invention has been described with respect to specific modifications, the details thereof are not to be construed as limitations, for it will be apparent that various equivalents, changes and modifications may be resorted to without departing from the spirit and scope thereof and it is understood that such equivalent embodiments are to be included herein. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

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